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A REVIEW OF METHODS FOR THE CHEMICAL ANALYSIS OF ROTENONE-BEARING PLANTS

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INTRODUCTION

An attempt has been made to collect all references to methods used for the chemical analysis of rotenone-bearing plants, particularly of derris and cube roots. The compilation has been confined to articles describing methods or critically comparing results obtained by different methods. References giving only results of analysis or reviews containing no original contributions or critical observations have not been included. Likewise methods of utilizing the results of chemical analysis, directly or through various calculations, to obtain a measure of insecticidal effectiveness are not treated here. This problem is itself sufficiently distinct and important to require separate treatment.

The methods discussed here have been grouped primarily according to the substances which they determine. This classification, although difficult because of uncertainty as to just what substances are determined by many of the methods, was believed to be the most useful. At the end of the sections on methods for determining the more important constituents, a brief statement has been made of the present status of methods for the purpose. In the last two sections are grouped certain methods that give more or less empirical values, which it is difficult or impossible to interpret in terms of actual substances present in the sample.

SAMPLING

In this section only those few articles are reviewed which give specific details on the sampling of derris or cube root for analysis.

Georgi and Teik (34) in 1933 suggested a procedure to be followed in selecting a sample for analysis from a shipment of derris root.

In 1936 these authors (35) elaborated the method somewhat, so that, for the preparation of fresh roots, the procedure was as follows:

> "Fresh roots are sun-dried until they can be broken without exudation of plant-juices. The semi-dried roots are then cut into pieces varying from 1/4 to 1/2 inch long and the chopped material is further sun-dried until of constant weight. The material is then quartered for analysis until approximately 1/4 lb. remains. One hundred grams are weighed, then ground in a drug mill. The root is first passed through a disintegrator having spaces 1/32 inch wide, the spaces being approximately 1/4 inch apart. The material is ground a second time, the disintegrator being replaced by a 1 mm. sieve. The small amount of woody matter remaining is ground separately in a small mill and incorporated with the main sample. To obtain a representative sample of the ground root for analysis, the various fractions are shaken together in a jar provided with a closely-fitting lid, and the required amount of material is weighed before segregation of the fine particles has taken place."

In sampling small shipments it was proposed that 1 pound of roots be withdrawn at random from a bale of 200 pounds. In the case of a large shipment it was suggested that 10 percent of the bales be sampled and 5 percent by weight of each of these bales be removed. The roots were to be chopped into short lengths and quartered until 1 pound remained.

Cahn and Boam (13) in 1935, in an article on the determination of rotenone, made the following statement in regard to sampling:

> "Derris root should pass entirely through a 50-mesh sieve before it is sampled. Root, as ordinarily ground, frequently contains a proportion of coarse, spicular material, through which the powder (often extremely fine) tends to filter, thus causing an undue amount of coarse particles to be present in the top layers. If the amount of spicular material is considerable, the whole batch should be sieved through a 50-mesh sieve, and the samples taken from proportionate amounts of the fine and coarse material."

Cahn (12) again in 1936 stressed the necessity of proper sampling. He found that, of 10 bales analyzed out of a consignment of 40 bales from the same estate, the total extract ranged from 15 to 25 percent. The only satisfactory method of sampling derris root was said to be to grind it first, mix it thoroughly, and then analyze the mixed fine powder.

Georgi (31) in 1937 described a method used in sampling kiln-dried root during the process of baling. When half the quantity required for a bale had been loaded into the press, about 2 pounds of the root was taken at random from the heap and set aside as a sample from that bale. When 20 samples, say 40 pounds, had accumulated, the bulk sample of root was mixed, spread out in a thin layer on the floor, and quartered until about 1 pound remained. This amount was cut into 1-inch lengths and quartered until about 3 ounces remained. This quantity was further cut into 1/4-inch lengths and ground in the laboratory mill for analysis. In one case 25 samples covering 497 bales were analyzed.

In 1937 Levallois (82) stated that the sampling of rotenone-bearing roots was very delicate, as the rotenone content of a sample depended on the abundance of long fibers. Progressive grinding of roots gave fractions with rotenone contents as follows: Friable parts and short fibers 6, medium parts 4 to 4.5, and long fibers 3 to 4 percent.

This author did not take samples of derris or cube at random, but classed root in three or four categories according to size and sampled accordingly. From each lot he took an aliquot part of 1/10 to 1/20 and chopped up these parts. From each part he took a new fraction. These fractions made up the samples for analysis.

Chevalier and Chevalier (20) recommended that, since large and small roots differ in rotenone and total-extract content, samples taken for analysis should have about the same proportion of large and small roots as the whole lot.

Krukoff and Smith (80) in 1937 reported that, if a lot of crude Lonchocarpus roots is a mixture of different species, no sample will accurately represent the entire lot. In sampling large, unbaled lots of a single species, fairly accurate results were obtained by making a careful ocular estimate of the weight of small, medium, and large roots in a given lot and taking a corresponding sample. For smaller lots, such as roots from a single plant, the different sizes were sorted out, weighed, and the sample was taken accordingly. This method would be impractical for sampling baled shipments as they arrive in this country. In the preparation of samples of fresh roots for analysis more uniform results were obtained by drying them to about 12 percent moisture before reducing them to slices or shavings.

Guillaume and Hervé (53) in 1939 emphasized the importance of obtaining a representative sample in the analysis of plants containing rotenone. They suggested that a sample of at least 1 kg. of root be taken for grinding. The ground material should be mixed and sifted and then mixed again at the moment of sampling.

The Puerto Rico Experiment Station (123) recently reported that the already difficult problem of sampling baled derris root was further complicated by the variation in rotenone and total-extract content of roots of the same diameter found in work at this station. It was suggested that a representative sample of the baled product could be obtained by using a small tube with a rotary cutter at the sampling end. Such a tool would make it possible to take a fairly large number of small samples and thus remove from the bale the minimum amount of root material required for a reliable analysis.

Information on sampling was recently furnished by four large importers of derris and cube root: McCormick and Company, S. B. Penick and Company, John Powell and Company, Inc., and Derris, Incorporated (private communications). These firms agreed on the matter of sampling about 10 percent of the shipment, although S. B. Penick and Company stated that frequently half, or even all, the bales in a shipment were sampled. John Powell and Company stated that where possible they preferred to grind at least one-tenth of the shipment. Two sources described the methods employed in removing samples of whole root. Derris, Incorporated, stated that 10 percent of the bales were opened and small bundles of root picked out at random from various sections of each bale. This material was ground, mixed, and a 1-pound sample drawn for testing. McCormick and Company removed from each of the bales chosen three small bundles of root, and from each bundle chopped sections about 4 inches long from the fine ends, the large ends, and the middle. These sections were used to make a composite sample for analysis.

Discussion

The sampling of derris and cube roots for analysis is very important. The proper sampling of whole root is extremely difficult, and it is doubted if any method is entirely satisfactory. In addition to differences in rotenone and total-extract content of different roots in a shipment due to differences in plant source, it has also been shown that fine and coarse

roots of both derris and cube vary in their content of rotenone and total extract (32, 33, 63, 64). Hence, in sampling a whole-root shipment care must be taken not only to draw from different parts of the bale or case, but also to obtain a sample having approximately the same proportion of fine and coarse roots as does the whole shipment. This procedure was recommended by Chevalier and Chevalier (20) and by Krukoff and Smith (80). The method cited above as used by one American importer is an attempt to accomplish this.

The proper sampling of powdered root is no less important, although less difficult. Cahn and Boam (13) and other workers have pointed out that marked segregation of fine and coarse particles occurs on standing. In addition to Levallois (82) and others, Koolhaas and Meijer[1] have shown large differences in the rotenone and total-extract content of fine and coarse powder from a single sample. They found in a sample prepared by them that the material passing an 80-mesh sieve analyzed 9.8 percent of rotenone and 19.6 percent of total ether extract, while the coarse material, which did not pass the sieve, had 1.3 percent of rotenone and 4.0 percent of ether extract. This variation indicates the importance of correct sampling of powdered root, particularly material that has been subjected to shipment.

MOISTURE

In this section are discussed only procedures for the determination of moisture. Methods of drying the sample before extraction, which are not actual moisture determinations, are discussed in connection with the various rotenone and total-extract methods.

Tattersfield and Roach (122) in 1923, in determining moisture in samples of Derris elliptica, dried the materials to constant weight at the temperature of boiling dichloroethylene (55°-58° C.) in a partial vacuum over phosphorus pentoxide. Elevated temperature was avoided, as the powdered root was said to decompose on prolonged heating.

A similar method was used by Georgi and Curtler (32) in 1929 in analyzing derris roots.

Spoon (115) in 1931 determined moisture in derris by heating 5 grams of the ground root at 100°-102° C.

Georgi and Teik (34) stated in 1933 that moisture determinations might be made either by drying the finely ground material to constant weight in a steam oven at 100° C. or by distilling with xylene. In the former method a 5-gm. sample was used, while in the distillation method 40 to 50 gm. was employed. The distillation method was preferred, as it was said to eliminate oxidation during drying.

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[1] Report of the analysis of a sample of derris root by various laboratories. 19 pp., typewritten. Jan. 19, 1937.

Tattersfield and Martin (120), in their work on evaluation of rotenone-containing plants in 1935, reported determinations of moisture by these two methods. The xylene-distillation method was used in Malaya, while the same samples were analyzed for moisture in England by drying at 100° C. Higher results were obtained in Malaya, but the authors attributed the difference to greater absorption of atmospheric moisture there.

Again in 1936 Georgi and Teik (35) stated that moisture was best determined by the xylene-distillation method, but could also be determined by drying to constant weight at 100° C.

Koolhaas and Meijer[2] in 1937, in a report on the analysis of a sample of derris root by various laboratories throughout the world, gave the following results for moisture as determined by the several laboratories:

Laboratory	Method	Moisture Percent
A--United States Department of Agriculture (Jones)	Dried at 103-104° C. for 2 hours	6.1
B--Department of Agriculture, Straits Settlements and Federated Malay States (Georgi)	Xylene distillation	11.3
C--Commercial Museum of the Colonial Institute, Amsterdam (Rowaan)	Xylene distillation	10.4
D--The Cooper Technical Bureau, London (Cahn and Boam)	Dried at 100° C.	9.4
E--Rothamsted Experimental Station (Tattersfield and Martin)	Dried at 100° C.	9.3
F--Seil, Putt, and Rusby, New York (Seil)	Not known	9.9
G--Caeser and Lorentz, Halle-Saale, Germany	Over calcium chloride for 2 days	5.1
H--Diethelm, Ltd., Singapore	Dried at 85° C.	7.2
I--Laboratory for Chemical Research, Buitenzorg, Java (Koolhaas and Meijer)	Dried at 105° C., constant weight	9.6

[2] See footnote 1.

They concluded that moisture determination should be made by drying a 3-gm. sample at 105° C. to constant weight. The xylene or heptane method gave higher values, but the higher temperature to which the material was exposed was thought to lead to decomposition, and the method was not recommended.

Jones and Graham (71) in 1938, in the analysis of a large number of derris, cube, and timbo roots, determined moisture by drying 2-gm. samples at 106° C. for 2 hours. Two days' additional drying of some of the samples caused no significant additional loss in weight.

Results for moisture content of derris root by heating in the oven and by toluene distillation were compared in a report of collaborative work by the Imperial Institute and the Rothamsted Experimental Station (59). Values by the oven method were only slightly lower than those by the distillation method.

Meijer and Koolhaas (89) in 1940 determined the moisture content of powdered derris root by drying a 2- to 3-gm. sample to constant weight at 105° C.

Of some interest in connection with the determination of the moisture content of derris powder is the hygroscopicity of the material. Meijer (87) in 1938 studied this question by keeping samples at different relative humidities at approximately room temperature and measuring the increase in weight. After standing at 75 percent relative humidity roots originally having 6 to 8 percent of moisture were found to contain 11 to 12 percent of moisture, while at 95 percent relative humidity these roots had about 24 percent of moisture. He concluded that powdered derris root was "freely hygroscopic."

Discussion

It may be concluded from the references given that the most suitable method for determining moisture is the drying of a 2- to 5-gm. sample at about 105° C. to constant weight. This should require only a few hours in many cases, but overnight drying before the first weighing should be a good practice.

TOTAL EXTRACT

The determination of the total-extract content of derris and cube roots, although a comparatively simple procedure and one that has been made from the time of the earliest work on these materials, is nevertheless subject to considerable variation.

Tattersfield and Roach (122) in 1923, in the course of an investigation of *Derris elliptica*, pointed out that the following factors are to be considered in the determination of total extract:

(1) The extraction solvent must be selective.

(2) The root must be ground to an almost impalpable powder.

(3) Extraction must take place at fairly low temperatures; otherwise sparingly soluble compounds are formed.

(4) The extract undergoes chemical change on drying.

(5) The type of extraction flask may modify results.

(6) The extract is freed from the last traces of solvent with great difficulty.

They proposed the extraction of the root in a Soxhlet apparatus, using a flat-bottomed flask, with ether (dried over anhydrous calcium chloride and sodium), and drying the extract as rapidly as possible to constant weight of 100° C.

A similar method was used by Georgi and Curtler (32) in 1929. They also pointed out that slight decomposition took place on drying the extract, and the results were slightly low.

In the 1929 report of the Colonial Institute of Amsterdam (77) it was stated that when a large sample of root had been ground once to a coarse condition, a portion of this sample ground again to a finer state, and some of the latter ground a third time to a very fine powder, the amount of ether extract was found to be higher as the root was more finely ground.

Dodwell and Company (private communication) in 1930 outlined the method in use in the trade for determining total extract of derris root. Ten grams of powdered root was dried in a vacuum over sulfuric acid and extracted exhaustively in a Soxhlet apparatus with dry ether, free from alcohol. The powder was then reground in a mortar and extracted for an additional 4 hours. The extract was dried for about 1/2 hour in a water oven. Later Dodwell and Company (private communication) quoted a revised method for total extract in which the root was dried at 100° C. to constant weight and then extracted with dry, alcohol-free ether until exhausted. No second treatment was used. The extract was dried to constant weight at 100° C.

Total ether extract was determined by Spoon (115) in 1931 by extracting 5 gm. in a Soxhlet with absolute ether for 15 hours and drying the extractives at 100°-102° C.

Koolhaas's (78) ether extraction method for rotenone included the determination of total extract. The filtrate and washings from the rotenone separation were evaporated, heated at 80° C. in a vacuum for 1/2 hour, dried in a desiccator, and weighed. Their weight was added to that of the crude rotenone.

The method for rotenone proposed by Jones (62) in 1933 and involving extraction of the root with carbon tetrachloride included a procedure for total extract, by which the filtrate from the rotenone separation, upon evaporation, was dried 1 hour at 105° C. and its weight added to that of the separated rotenone.

Georgi and Teik (34) in 1933 compared other solvents with ether for the determination of total extract of derris root. Petroleum ether was unsatisfactory, since its solvent action was so low. Acetone gave a higher value than ether. Slightly higher figures were obtained with chloroform, and slightly lower results with carbon tetrachloride than with ether. Preliminary drying of the root did not change the amount of ether extractives obtained.

In a comparison of chemical composition and toxicity to insects of rotenone-bearing plants Jones, Campbell, and Sullivan (69) in 1935 determined total carbon tetrachloride extractives of several samples of derris and cube by the method of Jones (62). Acetone and benzene extractives were prepared by Soxhlet extraction of 10- to 20-gm. samples for 7 to 8 hours. In general the amount of material extracted with acetone was higher than that with carbon tetrachloride, while that with benzene was slightly lower.

Tattersfield and Martin (120) in 1935 gave results of the determination of total ether extract on several samples of derris. In the extraction of 5-gm. samples with ordinary ether slightly higher results were obtained in Malaya than in England. The results that were obtained with sodium-dried ether in England were slightly lower than those obtained by the use of ordinary ether.

In proposing their trichloroethylene extraction method for rotenone, Cahn and Boam (13) in 1935 stated that total extract could be determined by evaporation of the trichloroethylene solution to dryness and heating to constant weight in an oven at 100° C.

In 1936 Georgi and Teik (35) gave directions for the determination of ether extract of derris root. Five grams of the finely powdered root was extracted with ether in a Soxhlet for 16 hours. No appreciable additional extract was recovered when the marc was reground with sand and again extracted with ether. The ethereal solution was filtered if necessary to remove traces of suspended matter, the solvent distilled off, and the residue dried to constant weight in a steam oven. Constant weight was usually reached in 6 hours.

Meijer (85) in 1936 proposed a colorimetric method for the approximate determination of the total extract of derris root. This method is described in the section on Miscellaneous Colorimetric Determinations.

Worsley (132) in 1937 determined total ether extract by extraction of a 10-gm. sample in a Soxhlet for 24 hours and subsequent drying of the extract to constant weight at 100° C.

Guillaume and Proeschel (54) in 1937 determined total ether and acetone extract of derris and other plants by treatment of 5-gm. samples in a specially designed continuous percolator. Only 150 cc. of solvent was required, and the extraction was said to be complete in 12 hours. Extractions with chloroform and carbon tetrachloride were made in a continuous percolator of different design. All extracts were filtered, concentrated to about 20 cc., placed in the refrigerator until the solvent

evaporated, and then dried at 50° C. for 12 hours and weighed. Values for acetone extracts were highest, those for ether and chloroform lower and about equal to each other, and those for carbon tetrachloride generally lowest.

Koolhaas and Meijer[3] in 1937 reported on the analysis of a sample of derris root by several laboratories in various parts of the world. Some of the methods and results for total extract were briefly as follows:

Laboratory	Method	Total extract (moisture-free basis) Percent
A--United States Department of Agriculture (Jones)	25 gm. benzene, 2 periods totaling 24 hours	18.2
B--Department of Agriculture, Straits Settlements and Federated Malay States (Georgi)	5 gm. ether, 18 hours	19.3
C--Commercial Museum of the Colonial Institute, Amsterdam (Rowaan)	5 gm. ether, 48 hours	19.1
D--The Cooper Technical Bureau, London (Cahn and Boam)	10 gm. ether, 3 periods totaling 96 hours	18.0
E--Rothamsted Experimental Station (Tattersfield and Martin)	5 gm. Analar ether, 3 periods totaling 30 hours	18.9
	5 gm. anhydrous ether, 2 periods, totaling 16 hours	17.7
F--Seil, Putt, and Rusby, New York (Seil)	5 gm. ether, until exhausted	19.2
G--Caeser and Lorentz, Halle-Saale, Germany	5 gm. ether	18.8
H--Diethelm, Ltd., Singapore	25 gm. ether	18.1
I--Laboratory for Chemical Research, Buitenzorg, Java (Koolhaas and Meijer)	50 gm. ether, 2 periods totaling 48 hours	19.6

[3] See footnote 1.

The authors also reported their own results on 81 samples of derris. Results using ether were in close agreement with those using benzene, but the time required for exhaustive extraction with ether was 2 days and 3 nights, whereas benzene required only 36 hours.

In discussing the determination of total extract Meijer (86) in 1937 recommended slow percolation of derris powder with ether in a Soxhlet. This was accomplished by placing the powder in the extraction tube of the apparatus without using a thimble. He also stated that drying of the extract, after removal of most of the solvent, was better accomplished at 40° C. under reduced pressure than by the usual drying at 100° C.

Jones and Graham (71) in 1938 determined total benzene extract by Soxhlet extraction of 5-gm. samples.

The determination of total-extract content was studied by Jones and Sullivan (76) in 1938, in an endeavor to select the solvent and method that would most readily extract all the toxic substances from derris and cube roots together with the least amount of nontoxic material. The total-extract content of several samples was determined by several procedures with various solvents. Successive extracts of some of the marcs with acetone, methyl alcohol, and water were tested against mosquito larvae. In 7-hour Soxhlet extractions of 5-gm. samples the percentages of material extracted by benzene, carbon tetrachloride, and ether were generally lower than by chloroform, ethylene dichloride, trichloroethylene, and ethyl acetate, which gave values of about equal magnitude. Acetone generally extracted more material and methyl alcohol considerably more than did the other solvents. When samples were extracted with ether or benzene for an extended length of time, the amount of material removed agreed with that extracted by chloroform. Acetone extracts of marcs from extraction with chloroform were in general nontoxic. Methyl alcohol and water extracts following this were completely nontoxic. Acetone extracts of marcs from benzene and ether extractions were toxic. Thus, of the solvents tested, chloroform appeared to be the most satisfactory from the standpoint of selective extraction of the toxic material. Results by the multiple-extraction procedure and by the aliquot procedure with chloroform at room temperature were in agreement with those by Soxhlet extraction. Because of its convenience, particularly when rotenone was to be determined by the same method, the aliquot procedure was suggested as the most suitable for determination of total-extract content.

Rowaan and Van Duuren (108) in 1938 suggested chloroform extraction at room temperature for the determination of total-extract content of derris and cube root.

Meijer (87) in 1938 showed the effect of drying the sample at elevated temperatures on the amount of ether extract obtainable. Thus, a sample of powdered derris root which before being heated contained 23.6 percent of ether extract gave 19.1 percent after being heated at 60° C. for 2 hours and only 14.4 percent after being heated at 80° C. for 2 hours. He concluded that heating above 50° C. before analysis should be avoided.

Braak (9) in 1939 reported on the analysis of a sample of derris root analyzed by the Laboratory for Chemical Research in Java; by Seil, Putt, and Rusby in New York; and by Salamon and Seaber in London. The three laboratories obtained almost identical values for the ether-extract content.

In collaborative work carried out between the Imperial Institute and Rothamsted Experimental Station (59) the ether-extract contents of three samples of derris root were determined. Values obtained at the former laboratory, where a 16-hour extraction was made, were about 1 percent higher than those obtained at the latter station, where two 8-hour periods were used. By the latter scheme little additional extract was obtained in the second 8-hour period except in the case of a high-rotenone root, which gave 1.4 percent additional material. All values ranged from 25 to 30 percent of total extract.

Meijer and Koolhaas (89) in 1940 described the method used in their laboratory for determining rotenone by extraction of a 50-gm. sample for 65 hours with ether. The determination of total-ether extract was made in conjunction with this as follows:

> "The ether is distilled off (from the mother liquor from rotenone separation) on a water bath and the last traces are removed in a vacuum in a water bath not exceeding 40° C. The contents of the flask are blown up to a voluminous mass, and placed in a desiccator over lime for 2 days, after which time constant weight has been reached.
>
> "The difference in weight between the flask with resin and the empty flask gives the amount of resin. To this the amount of crude rotenone is added, giving the ether extract in 50 g. of the sample..."

These authors stated that the total-extract content was about the same when benzene, chloroform, or ether was used; hence the extra determination of ether extract was unnecessary when chloroform or benzene had been used as extraction solvent.

A scheme of automatic hot percolation was described by Martin (83) in 1940. The apparatus consisted of a glass liner fitted into a wider glass tube connected with a 250-ml. flask and a condenser. The powdered root was supported in the liner on a pad of cotton wool, and rapid extraction at the boiling point of the solvent was said to be effected. The percentages of resin extracted by this method with several solvents from 25-gm. portions of a sample of Derris elliptica were determined. With most solvents extraction was reasonably complete in 2 hours. Solvent efficiencies of a similar order to those given by Jones and Sullivan (76) were found. Ethyl acetate, ethylene dichloride, and chloroform were equally effective in extracting the toxic principles with a minimum of extraneous matter. The method described was said to have the advantages over the percolation method of Worsley (130) of being automatic and requiring little more than 150 ml. of solvent.

Graham (47), in his 1938 report to the Association of Official Agricultural Chemists, gave results of collaborative analyses of one sample of derris and one of cube root. Results for total ether extract were in fair agreement and ranged from 13.7 to 14.5 percent for the derris and from 21.1 to 23.3 percent for the cube sample. Details of the method were not given. In his 1939 report to the same Association Graham (49) recommended that the method for total ether extract used in the 1938 work be adopted as official. In a discussion (48) of insecticide analysis before the 1939 meeting of the National Association of Insecticide and Disinfectant Manufacturers, he described the method briefly. The method (2) was given in detail in the Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists. It is as follows:

> "Extract 5 g. of finely powdered root in a Soxhlet or other efficient extraction apparatus with ethyl ether for 48 hours. After extraction, concentrate extract and filter off any insoluble material that may be present. Receive filtrate in a tared beaker, evaporate off ether on steam bath, and dry in oven at 105° C. to constant weight."

Details of a modification of this method to be used on derris and cube powder in the presence of sulfur have been furnished by the Agricultural Marketing Service (124). The total ether extract obtained essentially as in the official method is weighed and then treated as follows:

> Add 25 cc. of acetone (which has previously been saturated with sulfur at room temperature), disintegrate the residue with a stirring rod, and stir until the plant resins are in solution; filter, under suction, through a weighed Gooch crucible fitted with a disk of filter paper, rinse out the sulfur from the beaker, and wash with acetone (saturated with sulfur). Allow the crucible to remain under suction for 5 minutes, and then place in an oven at 105° C. for 15 minutes. Cool and weigh. The weight of this sulfur residue, subtracted from the weight of the residue in the original beaker, represents the ether-soluble plant extract material in the sample.

Among commercial firms handling derris and cube root who recently furnished information on methods of analysis (private communications), McCormick and Company, S. B. Penick and Company, and John Powell and Company used procedures for total extract essentially similar to the official method. S. B. Penick and Company also used total chloroform extract and found this determination more convenient to handle. Derris, Inc., used the ether extract determination only as a check and ordinarily determined total extract in conjunction with analysis for rotenone. The method used by this firm for determining rotenone, described in the section on Rotenone, involved extraction of the sample with acetone and crystallization of the rotenone from carbon tetrachloride. The mother liquor from the rotenone separation was then treated as follows:

> The nonrotenone portion (from a 25- to 50-gm. sample) in carbon tetrachloride is concentrated to a small volume, and the small amount of carbon tetrachloride remaining is removed

by distillation with about 25 cc. of isopropyl alcohol. Usually three 25-cc. additions of isopropyl alcohol are made before the resins are finally brought to constant weight by heating the tared distillation flask containing them in a vacuum oven at 80° C.

Results by this method were said to check with those by ether extraction.

Discussion

Undoubtedly the most generally used method for determining total extract of derris and cube roots is extraction with ether. The principal disadvantage in the use of this solvent is the great length of time required to obtain complete extraction. As a matter of fact any solvent that gives comparable values may be used for this purpose. Meijer and Koolhaas (89), Jones and Sullivan (76), and others have pointed out that that the amount of total extract obtained with chloroform agrees very well with that obtained with ether, and the time required for complete extraction is a matter of a few hours instead of days. The use of chloroform for determining total extract would therefore seem to be much preferred to the use of ether. Determinations of total extract in the laboratories of this Bureau are made with chloroform.

Unfortunately, the use of decolorizing carbon in the chloroform extraction method for rotenone, although a distinct advantage from the standpoint of rotenone determination, prevents the use of another aliquot for total extract as suggested by Jones and Sullivan (76). Graham (46) has shown that some of the extract is adsorbed by the carbon. However, it should be possible to devise a scheme in which the sample is extracted with chloroform without carbon and filtered as in the older rotenone method (72), a suitable aliquot taken for total extract, the remainder treated with carbon and filtered, and the proper aliquot taken for rotenone determination. A study should be made, however, to ascertain whether or not this scheme would involve loss of rotenone due to adsorption by the carbon.

ROTENONE

Methods designed to determine only rotenone are included in this section.[4] Many other methods, particularly colorimetric procedures, have been proposed for rotenone, but when applied to whole derris and cube roots or extracts they also determine additional materials. These methods are discussed in other sections of this review.

Geoffroy (29) in 1895 was the first investigator to isolate rotenone in a pure condition. Although not quantitative, the method of separation is of interest. It involved prolonged extraction of the plant material (cube) with petroleum ether and crystallization of the crude product from alcohol.

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[4] The solubility of rotenone in various solvents (73, 74) is of interest in connection with its determination.

Subsequent early investigators (Nagai (90), Ishikawa (60), and Takei (117)) generally employed ether for the separation of rotenone. Tattersfield and Roach (122) used alcohol. The same authors quote Durham as stating that the best method of obtaining pure rotenone was extraction with petroleum ether followed by recrystallization from alcohol, the method employed earlier by Geoffroy.

Roark (100) in 1930 was the first to propose a quantitative method for determining rotenone. This method, based on the separation used at that time in the United States Department of Agriculture, consisted essentially in extracting 100 grams of root with ether and crystallizing the rotenone from the extract concentrated to 25 cc. The filtered crystals were washed with ether, dried, and weighed.

Brown and Skinner (10), as editors of Wiley's Principles and Practice of Agricultural Analysis, quoted Roark's method. For the determination of rotenone in commercial derris preparations they suggested extraction with ether and precipitation of the rotenone, along with some resin, from the concentrated extract by the addition of petroleum ether.

Spoon (115), in the determination of rotenone in 12 samples of derris root, utilized crystallization from ether by Roark's method.

In 1931 Jones (61) suggested the use of carbon tetrachloride to replace ether in the analytical extraction of rotenone from derris and cube roots. Ether often gave extracts from which it was difficult to separate rotenone. Carbon tetrachloride extracts gave a quicker and more selective separation of the rotenone. In several cases in which no rotenone could be separated from the ether extract, carbon tetrachloride extracts separated rotenone readily. The rotenone separated from solutions in carbon tetrachloride as a solvate containing 1 mole of the solvent to 1 mole of rotenone.

Blackie (7) in 1932 described an apparatus with groundglass joints and mercury seals, which was especially designed for the determination of the rotenone content of a Fijian plant by extraction with ether. In the same year Blackie (6) published results obtained for the rotenone content of Derris uliginosa using this apparatus and extracting for 36 hours with ether.

Georgi and Teik (33) in 1932 described a modification of Roark's ether extraction method. They dried a 100-gm. sample in a water-jacketed vacuum drying oven at approximately 75° C. and then extracted with ether. The extract was concentrated to 40 cc. and crystallized in the cold cabinet overnight.

In the same year Koolhaas (78) discussed the methods for the estimation of rotenone then in use. The ether extraction methods in particular were said to be subject to error from such sources as insufficient fineness of root, too large a sample so that on evaporation of the extract the other substances present exerted an appreciable solvent effect, difficulty in washing the rotenone free of impurities, and difficulties encountered in the filtration of viscous ether solutions. He proposed an ether extraction method for derris root designed to overcome these objections.

As a measure of the purity of the crude rotenone the melting point was determined and, by means of a curve plotted from the melting points of mixtures of various percentages of residual extract and pure rotenone, the amount of pure rotenone in the crude crystalline material was obtained. This method with few modifications has been used in the Dutch East Indies since that time. The modified method now in use is described later in this section in connection with the more recent article by Meijer and Koolhaas (89).

In 1933 Jones (62) described a method of assaying derris and cube for rotenone using carbon tetrachloride for extraction and crystallization. The plant material was extracted in a Soxhlet with the solvent, and the extract concentrated and set aside to crystallize. The rotenone separated as its carbon tetrachloride solvate, containing equimolecular proportions of rotenone and solvent. The extract was cooled in ice, and then the crystals were filtered through a Gooch crucible and washed with ice-cold carbon tetrachloride. The crystalline material was dried to constant weight at room temperature and weighed. For more rapid but less accurate results the extract was crystallized in an ice bath for a few hours and the solvate, after separation, dried in an air draft. This early method has been superseded by the method of Jones and Graham (72), from which has been evolved the official method of the Association of Official Agricultural Chemists (2), which is given in detail later in this section.

If the sample contained over 5 percent of moisture, it was suggested that it be air-dried at not much over room temperature before extraction. Lower results for rotenone were obtained when samples of derris root were dried at 100° C. in a vacuum for 5 hours. Overnight (17 hours) extraction was recommended for most samples, as it was found that some samples giving 6 to 10 percent of rotenone in 8 to 10 hours gave results about 1 percent higher when extracted for 15 to 17 hours. As a means of checking the purity of the separated rotenone solvate, heating to drive off the carbon tetrachloride of crystallization and weighing the resulting rotenone were suggested. A determination of the optical rotation of the separated material was stated to give an indication of purity, while the chlorine and methoxyl contents were also suggested as of possible value for this purpose. The separated crystalline material was examined qualitatively to make certain that it was rotenone. This was done by microscopic examination and melting-point determination of the material recrystallized from amyl acetate.

Several samples that gave no rotenone by the earlier ether extraction and crystallization procedure gave as much as 2 percent by the carbon tetrachloride method. The latter method was believed to give correct results with roots containing over 0.5 percent of rotenone but with roots containing 0.3 per cent of rotenone or less it was inaccurate. For such material it was suggested that large samples be used. A scheme involving extraction with acetone and crystallization of the extract from carbon tetrachloride was tried. Although acetone gave a more rapid extraction of the rotenone, much of the acetone extract was insoluble in carbon tetrachloride. The additional manipulation necessary resulted in a less pure product.

Also in 1933 Georgi and Teik (34) studied the extraction and crystallization of rotenone and suggested a method involving the use of carbon tetrachloride. They suggested modifications to the ether extraction methods of both Roark (100) and Koolhaas (78) but considered that even with the modifications these methods did not give satisfactory results. One change introduced in Roark's method involved drying the root in a vacuum at 75° C. for 6 hours. They stated that the drying did not change the amount of extractives obtained, but believed that the presence of moisture interfered with the crystallization of the rotenone. The method suggested by these authors for estimating rotenone using carbon tetrachloride is briefly as follows:

> Fifty grams of the finely-ground root is treated for 72 hours (three active 8-hour periods) in a Soxhlet with carbon tetrachloride. The extract is concentrated until the solution begins to thicken, and is allowed to cool and seeded if necessary. The flask is allowed to stand in the cold cabinet for 24 hours. The crude solvate is filtered on a tared Gooch crucible and washed with 10 to 15 cc. of ice-cold carbon tetrachloride. After standing in the air for 24 hours it is weighed. The filtrate from the first crystallization is concentrated, cooled, and seeded. It is allowed to stand in the cold cabinet overnight and any additional crystalline material treated as with the first crop. The total crystalline material is treated with boiling alcohol, the solution cooled, and the separated rotenone dried at 100° C. and weighed. A correction is made for the solubility of rotenone in alcohol. The purity of the recrystallized product is checked by melting-point determination.

These investigators tried acetone and chloroform for extraction followed by crystallization from carbon tetrachloride. The separation of carbon tetrachloride-insoluble resinous material in the acetone extracts interfered with the separation of the rotenone, and the use of this solvent was not recommended. Chloroform proved satisfactory and extracted the rotenone in less time than did carbon tetrachloride, but any saving of time over direct treatment with carbon tetrachloride seemed doubtful. The rotenone values obtained by the chloroform and carbon tetrachloride methods were in close agreement. The values by the modified ether methods were in general lower and were irregular.

Georgi (30) in 1933 stated that carbon tetrachloride had been substituted for ether in the estimation of rotenone.

Spoon and Rowaan (116) in 1933 described the method used by them for determining rotenone in derris root. This was essentially the method of Roark with some modifications, such as longer crystallization and determination of the melting point of the separated rotenone. The latter step was designed, not as a means of calculating the purity of the material as in the method of Koolhaas, but as a check on its identity and approximate purity.

A procedure proposed by Takei, Miyajima, and Ono (118) in 1933 was a close approach to a truly chemical method for rotenone. Since the method

was designed to determine both rotenone and deguelin, it will be discussed only briefly here and described in more detail in the section on Deguelin and Rotenone Plus Deguelin. The root sample was extracted with ether and after crystallization of a first crop of rotenone from the extract the evaporated mother liquor was oxidized so that there were formed dehydrorotenone, from the remaining rotenone, and dehydrodeguelin. The mixture of insoluble dehydro compounds was separated and weighed. It was then subjected to catalytic hydrogenation under suitable conditions, when the dehydrorotenone was converted to the alkali-soluble isodihydrodehydrorotenone while the dehydrodeguelin remained unchanged, thus in effect giving separate values for rotenone and deguelin. Some derris-root samples gave surprisingly large amounts of additional rotenone by this method. In one extreme case only 0.56 percent of rotenone was obtained by crystallization, while an additional 4.74 percent was obtained by the chemical treatment of the mother liquor. On the other hand, in a sample giving 6.38 percent of rotenone by crystallization only 0.52 percent of additional rotenone was obtained. Values on some other samples ranged between these extremes. One sample giving no rotenone by ether crystallization gave 0.97 percent by the chemical method. Later work by Tattersfield and Martin (<u>120</u>), however, has shown that the ether crystallization used by Takei and coworkers was incomplete.

In the same year Danckwortt and Budde (<u>24</u>), in an investigation of methods for the evaluation of derris root, proposed the separation of rotenone by extraction with chloroform followed by crystallization from ether. The powdered root was covered with 10 times its weight of chloroform, and the mixture allowed to stand for 24 hours with frequent agitation. An aliquot of the chloroform extract equivalent to six-tenths of the original sample was filtered, the chloroform removed, and the residue crystallized from ether.

In 1934 Danckwortt, Budde, and Baumgarten (<u>25</u>) reviewed various crystallization methods for the determination of rotenone in derris and described other means of evaluation. The ether-extraction method of Koolhaas (<u>78</u>) was said to be too long. They considered the Jones (<u>62</u>) carbon tetrachloride method to be good only for rotenone contents above 4 percent. The method of Takei, Miyajima, and Ono (<u>118</u>) was said to be unobjectionable theoretically but very troublesome and time-consuming. The method of Danckwortt and Budde (<u>24</u>) was also mentioned.

Gstirner (<u>51</u>) in 1934 compared results by the polarimetric method with those by the ether-extraction and crystallization method. The much lower results by the latter method were explained as largely due to part of the rotenone remaining in the mother liquor.

Cahn and Boam (<u>13</u>) in 1935 made an extensive study of the determination of rotenone in derris root and resin. When derris resins were dissolved in carbon tetrachloride to separate the crystalline solvate, the solubility was said to be increased to an unusual extent by the presence of the resin. A maximum yield of rotenone was obtained when 2 cc. of carbon tetrachloride (saturated with rotenone) was used to dissolve each gram of resin. Room temperature was adopted for crystallization and filtration, as little difference was found between results obtained at 0° and

at 18° C. Excessive washing with carbon tetrachloride saturated with rotenone led to marked decrease in yield, with no increase in purity. In the determination of rotenone in derris roots 8 to 17 hours' extraction with carbon tetrachloride was not sufficient to remove all the resin or the rotenone. The authors preferred to use trichloroethylene, which usually gave complete extraction of rotenone in 8 to 12 hours. The rotenone was determined by evaporating the extraction solvent and crystallizing from carbon tetrachloride saturated with rotenone.

When pure rotenone-carbon tetrachloride solvate was stirred with 5 parts of alcohol saturated with rotenone, a quantitative yield of pure rotenone was obtained. Tests with mixtures of rotenone and "de-rotenonized" resin showed that a quantitative yield of the added rotenone was obtained from such a mixture when kept for 3 hours. It was later found advisable to keep such a mixture overnight. The alcohol recovery of the separated solvate from derris-root samples was said to range usually from 83.5 to 89 percent and rarely from 80 to 90 percent. Tests of purity by methoxyl content, chlorine content, and optical rotation gave values ranging from 89 to 95 percent on samples on which the alcohol-recovery values ranged from 84.5 to 89 percent. The question as to whether the purity of the solvate should be taken into consideration when a rotenone content was stated was considered by these investigators. The yield of rotenone was said to be lower than the amount actually present, and the amount of rotenone left in the mother liquor might be greater or less than the 10 to 15 percent impurity in the solvate. The value given was therefore only a minimum figure obtained by determining the yield and the purity of the solvate.

When an appreciable amount of pure rotenone was added to resins containing no rotenone by the usual procedure, an excess of rotenone over that added was obtained. The excess ranged from 7 to 15 percent of the resin in the samples tested. Resins of this kind were called "Sumatra-type" by the authors, and the rotenone obtained from them was termed "hidden" rotenone. The highest yields of hidden rotenone were obtained when 4 gm. of resin and 1 gm. of rotenone were dissolved in 10 cc. of carbon tetrachloride saturated with rotenone and the mixture was kept overnight and then filtered as in the usual procedure. Six samples of "normal" resins, containing appreciable proportions of rotenone, gave no significant amount of excess rotenone when treated in this way. However, a sample of root that had given a rotenone content of only 6.2 percent of the resin (1.5 percent of the root) as determined by the ordinary method gave 17 percent of rotenone in the resin when treated for hidden rotenone.

These authors stated that derris root should be dried, prior to extraction, in a vacuum desiccator to not more than 5 percent of moisture. The presence of much moisture was said to retard the rate of extraction and also to cause the development of acidity in chlorinated solvents. Drying at 100° C. caused decomposition and low results for rotenone. The procedure proposed by these authors was as follows:

A quantity of root sufficient to give 5 to 10 gm. of extract is extracted with trichloroethylene for 8 hours in a Soxhlet apparatus. The solvent is changed and the extraction continued for 4 hours longer. If the second solution is more than pale yellow, the extraction is repeated with fresh solvent for an additional 4 hours. The combined extract is evaporated until the extract becomes thick. A gentle current of air is then blown into the flask while the flask is rotated over a naked flame until the odor of solvent is replaced by the odor of derris resin. The flask is weighed to determine the approximate weight of resin, and 2 cc. of warm carbon tetrachloride, saturated with rotenone, is added for each gram of resin and the resin dissolved rapidly. The solution is cooled, seeded if necessary, and kept overnight. It is filtered by suction through a tared Gooch crucible containing a disk of filter paper, and the solvate is washed with carbon tetrachloride saturated with rotenone until the filtrate is nearly colorless. The crystalline material is dried to constant weight in air below 50° C. The purity of the solvate is tested by the alcohol-recovery method already described.

A method for determining hidden rotenone in resins poor in rotenone involved the procedure already described for this purpose. The authors concluded that the usual carbon tetrachloride method gave low results if the rotenone content of the resin was below about 17 percent, was seriously in error if it was below 10 percent, and failed completely for resins of very low rotenone content.

The determination of rotenone by extraction with carbon tetrachloride was described by Pozzi-Escot (93) in 1935. If the determination was made at room temperature, the sample was extracted for 18 to 22 hours. Hot extraction, which was recommended by the author, required only 6 to 8 hours. The rotenone was crystallized as the carbon tetrachloride solvate, separated, and weighed. With certain products it was found preferable to extract with acetone or ether. After the solvent had been evaporated, the residue was extracted with hot carbon tetrachloride and the crystallization conducted as before.

Later in this year the same investigator (94) suggested that extraction with acetone, alcohol, ether, or ethyl acetate considerably shortened the time required for this operation. The use of a "Shumagawa"[5] instead of a Soxhlet extractor was also said to permit more rapid extraction. The extract, freed of solvent, was treated with hot carbon tetrachloride, and the rotenone crystallized from this solvent.

Rowaan (104) in 1935 described the method for determining rotenone in derris and cube roots used at the Colonial Institute of Amsterdam. In condensed form it was as follows:

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[5] Evidently the apparatus of Kumagawa and Suto (81), which is also mentioned by Guillaume and Hervé (53).

> To 50 gm. of the plant material 250 cc. of chloroform is added in a 500-cc. beaker, which is covered with a watch glass and allowed to stand 6 hours, with occasional stirring. The chloroform solution is then filtered, and the mass on the filter is washed with 50 cc. of chloroform. The filter with the mass on it is replaced in the same beaker, and 100 cc. of chloroform is added. The mass is stirred again and allowed to stand overnight. Then the chloroform is filtered through a new filter and the mass washed with another 100 cc. of chloroform in two 50-cc. portions. The chloroform is evaporated completely from the combined filtrates, the last traces being removed by stirring the flask carefully while holding it over a naked flame and blowing a current of carbon dioxide through it. The extract freed from chloroform is dissolved in 20 cc. of carbon tetrachloride by boiling under a reflux condenser for several minutes. Upon cooling rotenone separates as rotenone-carbon tetrachloride solvate. When crystallization is retarded, the solution is seeded with a small quantity of solvate. The mass is kept overnight in an ice chest to ensure complete crystallization. The separated solvate is collected by suction in a tared (fritted) glass crucible, washed with about 15 cc. of carbon tetrachloride (saturated with rotenone) in small portions, and dried overnight to constant weight (in air at room temperature).

In another article in the same year Rowaan (105) compared results obtained on authentic derris samples by a modification of Roark's method (100) and by the polarimetric method of Danckwortt and his coworkers (25). He recommended the extraction methods of Roark or of Jones (62) or modifications of them as the most workable for the determination of rotenone.

In the course of a study by Tattersfield and Martin (120) in 1935 of the evaluation of derris root, determinations of rotenone were made on the same samples both in Malaya, the source of the material, and at Rothamsted, England. In Malaya rotenone was determined by the method of Georgi and Teik (34). At Rothamsted a 50-gm. sample was extracted with ether and the extract was freed of solvent and dissolved in carbon tetrachloride; the crude solvate obtained was recrystallized from alcohol. Rotenone was also determined by the trichloroethylene method of Cahn and Boam (13). In one sample an extraction was also made with chloroform. The values obtained for rotenone by these various methods were in general in good agreement. The rotenone content of the samples ranged from about 2 to about 10 percent. In working with the method of Takei, Miyajima, and Ono (118) the writers found that in some samples much less rotenone could be crystallized out from ether by the preliminary crystallization recommended by Takei and his coworkers than was separated by the Jones (62) method from carbon tetrachloride.

In further work on the evaluation of rotenone-containing plants, Martin and Tattersfield (84) in 1936 studied the effect of removing the toxicarol upon the separation of rotenone from carbon tetrachloride solutions of Sumatra-type and *Derris malaccensis* resins. It was found that,

with a Sumatra-type root from which no rotenone separated by the normal procedure, after most of the toxicarol had been removed by alkali treatment of the ether extract the rotenone separated readily when the residual resin was taken up in carbon tetrachloride. Two Sumatra-type roots from which no rotenone could be obtained by the usual method (without removal of toxicarol), gave about 2 percent of crude rotenone by Cahn and Boam's (13) procedure for "hidden" rotenone (adding excess rotenone). Purification of the crude solvate resulted in the greatly reduced values of 0.54 percent and 0.67 percent. Aliquots of resin from the same two roots were dissolved in ether, extracted with 5-percent potassium hydroxide, and the ether was recovered from the alkali-insoluble portion. The residual resin was dissolved in carbon tetrachloride and the solvate crystallized in the usual way. Values of 0.47 and 0.89 percent, respectively, of crude rotenone were obtained by this method, and these results were reduced to 0.40 and 0.66 percent, respectively, by purification. It thus appeared that in Sumatra-type roots the presence of large amounts of resinous material rich in toxicarol prevented the separation of the rotenone present, or treatment with potash removed some other inhibitor of crystallization. Furthermore, the solvate, which separated readily after treatment with alkali, was obtained in an amount agreeing closely with the figure obtained for the purified rotenone by the normal method. With Derris malaccensis root, although rotenone separated without the use of the hidden-rotenone technique, the product was very impure. Here again the alkali treatment gave a value for crude rotenone agreeing closely with that obtained for purified rotenone by the usual method. The authors admitted the possibility that the alkali caused some loss by inducing oxidation of the rotenone, but they suggested that an alkali pretreatment such as they described, if suitably controlled, might form the basis of a standard method of rotenone determination. In purifying the crude solvate by trituration with alcohol these investigators preferred to filter at 0° C., using alcohol saturated with rotenone at this temperature.

Beach (3) in 1936 proposed a serviceable method for determining rotenone based on extraction with chloroform, evaporation of an aliquot of the filtered extract, and crystallization of the rotenone from carbon tetrachloride. The method was essentially as follows:

> Shake 30 gm. of root with 300 cc. of chloroform in a stoppered 500-cc. flask at room temperature for 2 or 3 hours. Let stand overnight and then shake 1 hour more. Chill flask and filter contents rapidly into a suitable flask, observing precautions to prevent loss from evaporation. Adjust the temperature of the filtrate to that of the original chloroform and transfer a 200-cc. aliquot to a 500-cc. flask. Remove the chloroform by vacuum distillation, and treat with two successive portions of carbon tetrachloride, evaporating under vacuum each time to remove traces of chloroform. Dissolve the extract in 15 to 20 cc. of carbon tetrachloride saturated with rotenone solvate at refrigerator temperature. Induce crystallization of the extract in an ice bath and allow to crystallize overnight in the refrigerator. Cool the extract in an ice bath, filter through a Gooch crucible as in

other methods, and wash with carbon tetrachloride saturated with rotenone solvate at room temperature. Leave the crucible on the vacuum for 15 to 20 minutes and then weigh. Warm (not over 50° C.), again apply vacuum, and reweigh. Repeat this process until constant weight is attained.

Fine grinding of the sample was said to be unnecessary in this method; the root need be ground only to 20 mesh. It was stated that the method gave complete extraction of roots of high rotenone content.

In 1936 Robinson (101) proposed a method for estimating rotenone in haiaris from British Guiana, which involved extraction with carbon tetrachloride in a Soxhlet for 12 to 24 hours, crystallization of the concentrated and filtered extract for about 22 hours in a freezing chamber, and filtration of the extract after chilling to -10° C. The crystals were washed with carbon tetrachloride at -10° C., dried in a desiccator overnight, and weighed.

Georgi and Teik (35) in 1936 urged the adoption of a standard method for the estimation of rotenone and gave details of the method tentatively adopted in the Department of Agriculture of the Straits Settlements and Federated Malay States. The method was based on one that they had described earlier (34), the chief difference being that recrystallization of the crude complex from boiling alcohol was replaced by trituration with cold alcohol. Other modifications were as follows: (1) From 20 to 50 gm. of root was used, depending on the rotenone content, so that not more than 4 gm. of solvate would be obtained. (2) The extraction time was reduced to 16 hours by using two 8-hour periods and removing the sample and lightly grinding and mixing between these periods. The method was said to be satisfactory with all species of Derris in which the rotenone content exceeds 15 percent of the extract. With D. malaccensis, Kinta type (Sumatra type of Cahn and Boam), in which the rotenone content is about 2 percent, it was necessary to add sufficient rotenone to raise the proportion of that substance to about 30 percent of the total extract to induce crystallization. It was suggested that the final figure might be low because of rotenone remaining in the mother liquor and some passing into solution when the solvate was triturated with alcohol. The possibility of using the weight of the crude solvate and applying an appropriate correction factor was studied, but the purity of the solvate varied too widely, both in roots of the same and different species. Consequently the authors did not recommend the weight of the solvate as a standard on which to base the rotenone content.

Buckley (11) in 1936, in a study of the constituents of derris root, mentioned that long heating of extracts to expel residual solvent was to be avoided, as some change occurred which rendered subsequent crystallization of rotenone incomplete.

In this year also Worsley (130) made a study of the determination of rotenone in derris root and the bark of Mundulea suberosa. He described an apparatus for extracting the root by percolation with hot ethyl acetate, which comprised a percolator with a water jacket for maintaining an elevated temperature and a receiver immersed in cold water. With this apparatus

a 10-gm. sample of high rotenone content could be completely extracted with 200 cc. of hot ethyl acetate in about 45 minutes. Roots of lower content, of which larger samples were used, required more solvent and longer time but seldom over 2 1/2 hours. Worsley found it advisable to add sufficient rotenone before crystallization from carbon tetrachloride to bring the ratio of rotenone to total extract up to 40 percent. The purity of the carbon tetrachloride solvate prepared by the author's method was stated to range from 92.5 to 97 percent and averaged 94.7 percent, as judged by the alcohol recovery of Cahn and Boam. The rotenone obtained after the alcohol treatment was found by measurements of its optical rotation to range in purity from 98 to 99.9 percent, with an average of 99.2 percent. On the basis of the latter value the average purity of the crude solvate became 94 percent. Worsley proposed using this value in calculating the pure-rotenone content of a sample, unless particular accuracy was required. He also suggested the use of optical rotation to determine the purity of the solvate, stating that the carbon tetrachloride present had no effect on the rotation. Results by this method were slightly higher than by alcohol treatment. The amount of rotenone left uncrystallized in the resin was not great, as shown by cooling and by adding further large amounts of rotenone and crystallizing. Addition of 5 percent of charcoal to <u>Derris</u> root before extraction gave a lighter colored extract and a slight but definite increase in the purity of the solvate. Similar results were obtained when 10 percent of charcoal was added to <u>Mundulea</u> bark before extraction. The following is a condensed description of the method proposed by Worsley:

> Sufficient air-dried, ground material is taken to give about 1 gm. of rotenone, and 5 percent by weight of decolorizing charcoal is added for <u>Derris</u> or 10 percent for <u>Mundulea</u>. The mixture is run into the percolation tube, which is placed in the constant-temperature bath, maintained at a few degrees below the boiling point of ethyl acetate, and the lower end is fitted to a filter flask. Suction is applied by means of a water pump. The calculated amount of ethyl acetate is then heated almost to boiling and is poured into the top of the percolation tube. The amount required is approximately 20 cc. per gram when 10 to 20 gm. of material are used, 15 cc. per gram for 25 to 40 gm., 500 cc. for 50 gm., and 800 cc. for 100 gm. of material. As soon as the solvent appears at the bottom of the tube, suction is adjusted so that the rate of percolation is about 2 drops per second; when slightly more than half the solvent has come through, it is increased to about 4 drops a second; and finally, when practically all the solvent is through, full suction is applied.
>
> The extract is filtered into a distilling flask and practically all the ethyl acetate distilled off. The resins are transferred to a small, weighed beaker, and the ethyl acetate is removed on the water bath. The beaker is weighed to determine the amount of resins. Sufficient purified rotenone (40-mesh) is then added to bring the rotenone content in the resins up to at least 40 percent; in any case at least 1.0 gm. is added. It is stirred into the heated resins on a water bath, 2 cc. of

carbon tetrachloride saturated with rotenone for every gram of resins plus rotenone is added, and the mixture is warmed until solution is complete. The beaker is set aside until morning in a desiccator containing a dish of carbon tetrachloride; seeding is unnecessary. The crystals are filtered through a Gooch crucible on a disk of filter paper. They are washed with solvent (carbon tetrachloride-rotenone) until no further color is removed and dried for about 6 hours at about 40° C. The weight obtained times 0.719 gives the amount of crude rotenone.

The purity is most accurately determined by triturating the rotenone-carbon tetrachloride complex with absolute alcohol saturated with rotenone, 5 cc. for every gram of complex, and leaving overnight in a desiccator containing a dish of alcohol. The rotenone is collected in a Gooch crucible, washed with 30 to 40 cc. of the solvent, and dried at 100° for 6 hours. From the weight of rotenone thus obtained the amount originally added is deducted; the difference is the amount in the sample. The purity of this rotenone may be determined by weighing out between 0.48 and 0.50 gm., dissolving in a stoppered vessel in 10.00 cc. of pure benzene, and determining the angle of rotation in a 200-mm. tube. From a previously prepared curve, or from the formula $C = (\alpha - 1.428)/4.066$, the concentration of pure rotenone is obtained and the purity thus determined. The purity may be taken as being 99.2 percent, and this means that for rotenone contents above 6 percent a correction of -0.1 percent is made, but for contents below 6 percent no correction is necessary.

A less accurate result for pure rotenone can be obtained by determining the angle of rotation of the rotenone-carbon tetrachloride complex by weighing out between 6.8 and 7.2 gm. and dissolving in 10.00 cc. of pure benzene. As before, the concentration of pure rotenone is determined and the percentage purity of rotenone in the complex calculated; from this figure 2.6 is deducted and the difference used to calculate pure rotenone in the sample.

An even more rapid method is to assume the purity of the complex to be 94 percent and to calculate pure rotenone on this basis. Except for a few unusual derris samples results of sufficient accuracy for routine estimations are obtained.

In 1936 many commercial analysts of derris and cube roots were using a method for rotenone developed by H. A. Seil.[6] This method has not been published, and several variations have been brought to the attention of the reviewer. In this method 50 gm. of root was extracted with carbon tetrachloride in a Soxhlet for 5 to 7 hours, or longer if necessary. Before extraction 0.34 gm. of rotenone-carbon tetrachloride solvate was added to the extraction flask, and after extraction the extract was concentrated to 40 cc. Crystallization and filtration of the solvate were carried out as

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[6] Details of this method were obtained in 1936 in private communications from McCormick and Co. and John Powell and Co.

in other methods. The crystals were washed with a cold saturated solution of rotenone in carbon tetrachloride and allowed to air-dry to constant weight. The value for rotenone was calculated from this weight of crude solvate.

Rowaan (106) in 1936 again warned against the use of Danckwortt's (25) polarimetric method for the determination of rotenone and stated that the most reliable procedure was some modification of the extraction-crystallization method.

The appearance of microscopic crystals of rotenone was described by Pozzi-Escot (96) in 1936 as an aid in the identification of this material in analytical work.

In 1936 government agencies of the Dutch East Indies prepared a sample of derris root and sent subsamples for analysis to nine laboratories in various parts of the world. In 1937 Koolhaas and Meijer[7] reported the results of this investigation. Values for rotenone on a moisture-free basis were as follows:

Laboratory	Rotenone Percent
A--United States Department of Agriculture (Jones)	8.1 (pure)
B--Department of Agriculture, Straits Settlements and Federated Malay States (Georgi)	8.6 (pure)
C--Commercial Museum of the Colonial Institute, Amsterdam (Rowaan)	9.7 (crude)
D--The Cooper Technical Bureau, London (Cahn and Boam)	7.7 (pure)
E--Rothamsted Experimental Station (Tattersfield and Martin)	8.4 (pure)
F--Seil, Putt, and Rusby, New York (Seil)	7.5 (crude)
G--Caeser and Lorentz, Halle-Saale, Germany	12.3 (crude)
H--Diethelm, Ltd., Singapore	6.3 (crude)
I--Laboratory for Chemical Research, Buitenzorg, Java (Koolhaas and Meijer)	9.8 (pure)

[7] See footnote 1.

Various methods of extraction were used, but in all except two of the laboratories the rotenone was separated as the carbon tetrachloride solvate. Laboratory A used both Soxhlet extraction and maceration with refluxing; the latter method, using benzene, was preferred; the crude solvate was purified by an alcohol-recovery test. In laboratory B the sample was mixed with sand and extracted with carbon tetrachloride for two 8-hour periods; an alcohol-recovery test was included. The method used by laboratory C involved maceration with chloroform for two extended periods; no test of the purity of the solvate was made. Laboratory D made comparative Soxhlet extractions with trichloroethylene, chloroform, and ether; ether extraction gave markedly lower values, and the first method was preferred; alcohol recovery was used for purification. Ether extraction of the sample mixed with sand in a Soxhlet for 24 hours was used by laboratory E; the usual alcohol-recovery test was employed. Laboratory F extracted the sample for 6 hours in a Soxhlet with carbon tetrachloride and used no final purification. In laboratory G an extraction method using chloroform in a Soxhlet was compared with the optical rotation of a benzene extract of the sample; both methods gave results much higher than those obtained by other laboratories. Ether extraction in a Soxhlet for 8 hours, followed by crystallization directly from the ether, with no purity tests, was the method used by laboratory H. The laboratory of the authors of the report (laboratory I) used Soxhlet extraction with ether for 48 hours, followed by crystallization from the ether and measurement of the purity by determination of the melting point.

Koolhaas and Meijer criticized the results on the basis that many of the laboratories did not obtain complete extraction of the root. Benzene, chloroform, and ether, the last-named as used in their laboratory (I), were stated to be suitable extraction solvents, but longer extraction than was used by most of the laboratories was said to be necessary. Use of melting-point determination in judging the purity of the separated rotenone gave higher values than alcohol recovery, and these authors preferred this method. They also pointed out that the optical rotation of the whole extract is of no value in determining the rotenone content. They recommended that for analysis derris root should be ground so that at least 75 percent passes an 80-mesh sieve, and that the moisture content should not be over 12 percent.

Georgi and Teik (36) in 1937 stated that rotenone may be lost at two stages of its determination. Thus, some may not crystallize from the carbon tetrachloride but may remain in the mother liquor, while some may pass into solution when the complex is triturated with alcohol. Determinations of the optical rotations of alcoholic filtrates after recovery of rotenone indicated that the loss in triturating the complex with alcohol amounted to approximately 6 percent, calculated on the weight of rotenone recovered. It was not found possible to devise a method for estimating the amount in the carbon tetrachloride mother liquor, but the low optical rotation of the residual bodies in this liquor pointed to a smaller degree of retention than with the alcohol liquor. The total loss was estimated as possibly 10 percent.

Begtrup (4) pointed out in 1937 that extraction of derris or cube root with a low boiling solvent, such as ether in a Soxhlet apparatus, is incomplete. On the other hand, he stated that if a solvent of higher boiling point is used the prolonged heating generally destroys the extractive substances and thus renders crystallization of the rotenone difficult or impossible. To overcome these difficulties he recommended extraction with toluene at room temperature. The procedure suggested by Begtrup was as follows:

> Thirty grams of 100-mesh material is packed in an ordinary funnel, thoroughly moistened with toluene, and washed six times with 20 cc. of toluene (each extract is permitted to drain off completely). The combined extract is diluted to 150 cc., and a 50-cc. aliquot is taken. The toluene is distilled off on an oil bath maintained at about 130° C. The residue is dissolved in 7-8 cc. of carbon tetrachloride saturated with rotenone at 10° C. and transferred to a weighing bottle. Washings bring the total volume to 12-15 cc. Crystallization is allowed to proceed overnight at 10° C. The crystals are filtered, washed, and dried, as in other methods.

Chevalier and Chevalier (20) in 1937 described a method involving extraction with chloroform and crystallization from carbon tetrachloride, similar in all essential details to previous methods. The rotenone was purified by recrystallization from warm alcohol.

Ripert (99) used dichloroethylene for the extraction of rotenone.

The determination of rotenone in samples of Derris and Mundulea was described by Guillaume and Proeschel (54) in 1937. They used a modification of the method of Koolhaas (78). Extraction of the 5-gm. sample with ether in a continuous percolator was carried out as described in the section on Total Extract. The evaporated extract was then treated as follows:

> Add 15 cc. of cold ether to the extract in a tared crystallizing dish (A), cover, and let stand in the refrigerator for about 24 hours. The extract dissolves and the crystals of rotenone deposit. Decant the solvent into a second tared crystallizing dish (B). Rapidly wash the crystals with a little cold ether. Decant the wash liquid into the crystallizing dish (B). Keep this dish in refrigerator for 24 hours. Dry crystallizing dish (A) in oven at 50° C. for 1 hour and weigh. If crystals form in dish (B), decant the liquid, wash as before, dry dish and contents at 50° C., weigh and add to weight of rotenone in dish (A).

These investigators attempted to check the sensitivity of the crystallization method. They added to the powdered root of a species of Lebeckia that contained no rotenone by qualitative tests progressively increasing amounts of rotenone in the form of carbon tetrachloride solvate and analyzed the mixtures by the methods indicated. As shown in the following tabulation, very good recovery of the rotenone was obtained:

Rotenone added per 5 gm. (as pure rotenone)	Rotenone found (by ether method)
Gram	Gram
0.355	0.3451
.071	.0713
.071	.0683
.0355	.0348
.0355	.0378

They stated that when less than 0.0355 gm. of rotenone was employed values were too low. Determinations by chloroform extraction (5-gm. sample) and crystallization from carbon tetrachloride on some of these prepared samples, as well as on samples of Derris and Mundulea, gave results in good agreement with those by ether.

Levallois (82) in 1937 said that ethyl acetate and chloroform were the two best extraction solvents for use in the determination of rotenone. He emphasized that all trace of extraction solvent must be eliminated under vacuum before crystallizing from carbon tetrachloride. Levallois stated that the tendency was to abandon the colorimetric methods in favor of the gravimetric methods for rotenone.

Crystallization methods for rotenone were said by Schonberg (110) to be open to the objection that they were not applicable to very small amounts of rotenone, that the results were not in accord with the insecticidal activity, and that they were very laborious. It was stated that the carbon tetrachloride complex contained variable proportions of impurities and that its content of pure rotenone could not be deduced. Colorimetric methods were recommended.

Worsley (131) in 1937 stated that prior to the publication of the work of Cahn and Boam (13) he had found that addition of pure rotenone to extracts of Mundulea suberosa bark resulted in a considerably greater net yield of rotenone. He obtained higher yields of rotenone by adding decolorizing charcoal to the powdered bark before extraction with ether. It was necessary, however, to increase the time of extraction and better extraction solvents were tried. Percolation with hot ethyl acetate gave the most satisfactory results. Details of this method have been described (130).

Rowaan (107) in 1937 recommended the use of chloroform at room temperature for the extraction of rotenone. He stated that drying at 60° C. for 1 hour brought the rotenone-carbon tetrachloride solvate to constant weight. Rowaan tentatively recommended determination of the purity by alcohol recovery, although he said that this process needed improvement.

Meijer (86) in 1937, before a meeting of the Buitenzorg (Java) Experiment Station staff, discussed the evaluation of derris and reviewed methods for the determination of rotenone. Treatment of the mother liquor from rotenone separation by chromatographic adsorption, by formation of the hydrazine of rotenone, and by other methods was said to show as much

additional rotenone as 10 percent of the remaining extract. Of three methods of determining purity alcohol recovery was said to give the lowest values, the melting-point method the highest, and the polarization method intermediate values. Because of ease of handling the last two were preferred.

A method for rotenone using ethyl acetate for extraction was suggested by Pozzi-Escot (97) in 1937. Extraction was accomplished by boiling under reflux. The extract, concentrated to a sirup, was treated with sufficient activated charcoal to make a dry powder and then extracted with a known volume of carbon tetrachloride already saturated with rotenone. This extract was diluted with carbon tetrachloride if necessary. The extract was made to the original volume of the carbon tetrachloride solution and set aside to crystallize for 24 hours at the temperature at which the solvent was saturated with rotenone. The crystals were filtered and weighed in the usual way.

Seaber (111) in 1937 reported analyses of derris, barbasco, and timbo for rotenone content using carbon tetrachloride, chloroform, trichloroethylene, and ethyl acetate as extraction solvents. In the case of carbon tetrachloride the sample was extracted at least 16 hours, and during crystallization the extract was kept at room temperature for 2 days and then in ice at least 3 hours before filtering. The chloroform extraction method was essentially that of Beach (3). The error introduced by change in volume due to solution of the extract was found to be small and was shown to be almost exactly compensated for by the effect of evaporation during filtering. Instead of using a mechanical shaker in this method it was found that equally good results were obtained by allowing the sample to stand in chloroform overnight, shaking occasionally by hand the next day, allowing to stand over another night, and filtering the next day. Extraction in a Soxhlet with chloroform was tried, but the solvate obtained was less pure. The trichloroethylene method of Cahn and Boam (13) and the hot ethyl acetate percolation of Worsley (130) were also tried. Purity of the solvate was calculated by polarization in benzene, which gave results a little higher than those by the alcohol-recovery method.

In almost all cases the chloroform method gave higher results than did carbon tetrachloride, and in some cases the differences were large. The question as to whether these differences were due to failure of the carbon tetrachloride to extract the rotenone or to decomposition was studied. In one typical extraction 82 percent of the rotenone was obtained in the first 8 hours' extraction with carbon tetrachloride. However, with this solvent a limit seemed to be reached before all the rotenone was extracted out of the root. Decomposition of rotenone in boiling carbon tetrachloride was found to be a factor in lower results by this solvent. Boiling rotenone in this solvent for 16 hours gave a loss equivalent to about 0.2 percent on a 5-percent rotenone root, and for 72 hours a loss equivalent to about 0.5 percent on a similar root. It was stated that carbon tetrachloride cannot be relied upon to extract rotenone completely. Extraction by the ethyl acetate method gave results in agreement with those by the room-temperature chloroform method but the solvate obtained was less pure. Seaber recommended as the best method for commercial purposes extraction with cold chloroform, crystallization from carbon tetrachloride, and determination of purity by polarization, the result to be reported in terms of pure rotenone.

Seaber also investigated the possibility of making use of the maximum of the ultraviolet absorption curve to estimate the percentage of rotenone in the crude solvate. Extreme dilution was necessary, but nevertheless results close to those by polarization were obtained in some cases, although in others the values were too high. The method could not be used as a routine process for purity but might be useful in identifying and roughly estimating rotenone present in small amounts in mixtures. An attempt to estimate rotenone by this method in whole extracts of the root was not successful because of the effect of impurities.

Jones (66) in 1937 studied the crystallization of the rotenone-carbon tetrachloride solvate from extracts and proposed a modified procedure for this step in the rotenone determination. The proposed crystallization procedure was as follows:

> The solvent-free extract from a 25-gm. sample of root is dissolved in 25 cc. of carbon tetrachloride, cooled in an ice bath, and seeded with crystals of rotenone-carbon tetrachloride solvate. If only a small amount of crystalline material separates, an accurately weighed amount of rotenone is added, so that at least 1 gm. of pure rotenone is present. This extract and a wash solution having 0.27 gm. of rotenone for 100 cc. of carbon tetrachloride are maintained at 0° C. in an ice bath overnight. The crystals are then filtered in a tared Gooch crucible, washed with 6-10 cc. of ice-cold wash solution, and dried to constant weight at 40° C. One gram is treated with 10 cc. of alcohol saturated with rotenone at room temperature and set aside at this temperature for 4 hours. This material is filtered through a tared Gooch crucible, washed with about 5 cc. of alcohol saturated with rotenone at the same temperature, and dried to constant weight at 105°. Corrections are made of 0.07 gm. for the rotenone dissolved by the 25 cc. of carbon tetrachloride used and also for any rotenone added.

The purity of the solvate was found to depend principally upon the proportion of rotenone to total extract and upon the proportion of solvent used in crystallization. As the so-called "pure" rotenone obtained by the alcohol treatment was not entirely pure, and as its purity depended on the purity of the solvate from which it was prepared, it was desirable to obtain the solvate in as nearly pure a form as possible. For this reason the author preferred to crystallize the solvate from a larger proportion of solvent than that used by Cahn and Boam (13). More rapid conversion of the solvate to rotenone was obtained when 10 cc. of alcohol per gram of solvate was used rather than the 5 cc. proposed by Cahn and Boam.

A determination of the precision of replicate results on one sample of derris of about 4-percent rotenone content showed a standard deviation of ± 0.05 percent. A study was made of the accuracy of the proposed crystallization procedure when applied to extracts of both derris and cube roots with various proportions of rotenone to total extract. The method used assumed that the nonrotenone portion of the extract exerts only a retarding effect on the crystallization and has little or no actual solvent effect on the rotenone, an assumption that was indicated by all the

writer's work up to this point. On this assumption extracts of approximately known rotenone contents were prepared from large samples of roots of 4-percent rotenone content or over. The extracts were subjected to a preliminary crystallization in the usual way, and the solvate was removed by suction filtration. The filtrate was then made to a definite volume, and aliquots were taken of such a size as to be equivalent to 25 gm. of root samples. The amount of rotenone remaining in each aliquot was calculated from known solubility figures. To the dried aliquots amounts of pure rotenone varying from 0.2 to 2.0 gm. were added, each was treated with 25 cc. of carbon tetrachloride, and crystallization was carried out by the proposed method. The actual weight of pure rotenone obtained was used without correcting for solubility in the carbon tetrachloride. Hence from the known solubility of rotenone in carbon tetrachloride at 0° C., a loss of 0.07 gm. was to be expected. The values for rotenone present (amount calculated from solubility plus amount added) were plotted against the amount lost in crystallization (amount present minus amount actually recovered). In extracts with normal proportions of extractives other than rotenone, the loss of rotenone in crystallization was great when only small amounts were present, but decreased with increasing amounts of rotenone until at 0.06 to 1.0 gm. it became practically constant at approximately the loss to be expected from the solvent effect of 25 cc. of carbon tetrachloride. Similar results were obtained with extracts of the same roots prepared to have both abnormally high and abnormally low proportions of nonrotenone resins. When extracts with normal proportions of rotenone were allowed to crystallize for 48 hours, complete crystallization to a value approximately equal to the solubility was obtained when only about 0.6 gm. of rotenone was present.

An extract of a Sumatra-type derris root was subjected to the same preliminary crystallization except that sufficient pure rotenone was added to assure quantitative crystallization. The filtrate was treated in the same way as described for those of the 4-percent roots. When about 1 gm. of rotenone was present, the loss on crystallization was constant and approximately equal to the solubility loss; consequently these resins are similar to ordinary resins in their effect on rotenone crystallization. The author therefore believed that the "hidden" rotenone described by Cahn and Boam (13) was a result of the retarded crystallization obtained with any extract having a low proportion of rotenone to nonrotenone resins; the addition of rotenone merely hastened the crystallisation. The accuracy of the Cahn and Boam method, in which 1 gm. of extract is dissolved in only 2 cc. of carbon tetrachloride, was briefly studied and appeared to be equal to that of the author's method.

It was concluded that accurate results by the proposed crystallization method were obtained only when the rotenone present was equivalent to 4 percent of the root, or when sufficient rotenone was added to bring the amount present during crystallization above this value. For the extracts studied the method gave values which, in view of the precision, were not significantly different from the actual rotenone contents. Because of the widely varying composition of different samples of derris and cube root, no general estimate was made of the accuracy.

In 1938 Jones and Graham (71) studied methods for the quantitative extraction of rotenone from derris and cube roots. The following general methods were compared:

(1) Soxhlet extraction using carbon tetrachloride.

(2) Boiling-multiple extraction, in which the sample was refluxed with the solvent on the steam bath and then filtered by suction and the marc was treated twice with fresh solvent in the same way. Benzene, carbon tetrachloride, chloroform, ethylene dichloride, trichloroethylene, ethyl acetate, and the benzene-alcohol azeotropic mixture were tested as solvents by this method.

(3) Boiling-aliquot method, in which the sample was treated with a weighed amount of solvent, refluxed on the steam bath, cooled, solvent added to replace that lost, the extract filtered, and an aliquot taken. Only benzene was tried in this method.

(4) Room temperature-multiple extraction method, similar to method (2) but carried out at room temperature. Only chloroform was tried.

(5) Room temperature-aliquot method, substantially the same as that proposed by Beach (3). Chloroform, benzene, and ethyl acetate were tested.

After removal of the solvent, crystallization of the rotenone was carried out by the method proposed by Jones (66). The marcs from multiple-extraction methods were tested for rotenone by a qualitative color test.

Tests were made on a large number of finely powdered samples of derris, cube, and timbo roots and on one sample of Tephrosia virginiana root. Marcs from the benzene-boiling-multiple extraction method and the chloroform-room temperature-multiple extraction method showed practically complete extraction of the rotenone. Results for rotenone by these methods were in agreement with those by the chloroform-room temperature-aliquot method. The latter method was preferred by the authors because of its convenience. Various phases of the chloroform-room temperature-aliquot method were then studied. It was found that the time of shaking during extraction might be reduced to 2 hours without seriously affecting the results, but to ensure complete extraction overnight shaking was advised. Fineness of the sample was an important factor in obtaining complete extraction. Results on coarsely ground samples were in some cases 1 percent lower than on the same roots reground to a finer size. It was stated that, to give satisfactory extraction by the aliquoting procedure, coarse samples should be ground so that at least 95 percent passed a 60-mesh sieve. Samples containing a high ratio of rotenone to total extract were found to be more difficult to extract than those with lower percentages of rotenone. When the ratio of rotenone to total extract was about 40 percent or over, particularly in the case of derris roots, it was necessary to employ the chloroform-room temperature-multiple

extraction method to obtain satisfactory extraction. Cube roots in general were more readily extracted of their rotenone content than were derris roots. Preliminary drying was unnecessary, since the moisture content of derris and cube roots as received in the United States was found not to be sufficiently great to interfere with their analysis. In addition the results for rotenone and the purity of the solvate were lower when the root was dried either at 100° C. or at 50° under vacuum before analysis. In the chloroform-room temperature-aliquot method replicate results on a sample by a single investigator in general agreed within about 5 percent. The average difference between the results by the two authors was only about 3.5 percent.

As a result of this work Jones and Graham (72) in 1938 proposed a complete extraction and crystallization method for rotenone based on the room temperature-aliquot extraction using chloroform and the crystallization as proposed by Jones. The method with some modification has recently been adopted by the Association of Official Agricultural Chemists (49) and is described in detail later in this section. For roots of abnormally high ratio of rotenone to total extract, or in any case of doubt as to the completeness of extraction, the alternative room-temperature extraction with successive lots of chloroform was suggested.

Cahn, Phipers, and Boam (17) in 1938 discussed methods for determining the various constituents of derris extract. They did not agree with Jones (66) that the nonrotenone resins exert no solvent effect on the rotenone. They cited as experimental evidence one derris extract which gave 39 percent of rotenone by the usual method including correction for purity, but gave 42 percent of pure rotenone when a first crop was crystallized and the mother liquor was allowed to be adsorbed on charcoal and rotenone recovered from the numerous fractions obtained. Hence the authors believed that the rotenone content calculated from the crude solvate was, by a compensation of errors, closer to the correct value than results based on pure rotenone. They tested the effect of the removal of the alkali-soluble material as suggested by Martin and Tattersfield (84), and found that in a series of Sumatra-type extracts substantially the same results were obtained by this method as by the hidden rotenone technique of Cahn and Boam (13) in which excess rotenone is added. They also discussed results by the Goodhue modification of the Gross and Smith color test (40), which determines primarily rotenone plus deguelin (see section on Deguelin and Rotenone plus Deguelin). They found that with the numerous samples of derris extract studied (except Sumatra-type extracts) the following relation held: Goodhue value = percent rotenone + 22 ± 3, where the values are expressed as percentage of the extract and the rotenone value is that for crude rotenone. They stated that this relation had been used successfully in the inverse sense to determine approximate rotenone contents from Goodhue values, and was especially valuable for this purpose when only small amounts of material were available.

Rowaan and Van Duuren (108) in 1938 recommended room-temperature extraction with chloroform and removal of an aliquot for the determination of rotenone in Derris and Lonchocarpus roots.

Seaber (112) in 1938 published results of analyses of derris, timbo, barbasco, and cube roots by the room temperature-chloroform-aliquot extraction method of Beach (3) and the short-time (6 hours) carbon tetrachloride Soxhlet extraction of Seil (see p. 25). The first method almost invariably gave higher results for derris and generally lower results for timbo and barbasco, while results for cube were only a little higher by the first method. It was suggested that derris roots be assayed by the room temperature-chloroform method, while other roots be analyzed by both methods and the higher result taken. As in previous work, Seaber determined the purity of the solvate by polarization.

The following method for the isolation of small quantities of rotenone from oleaginous seeds was described by Guichard (52) in 1938:

> The sample is extracted in the cold with ether or petroleum ether. After evaporation of the solvent the oily liquid is extracted with 50-percent acetic acid in a separatory funnel until the last lot gives no color reaction for rotenone. This acid solution is extracted with ether and the ether evaporated. The residue is dissolved in about 10 cc. of 50-percent acetic acid, and the solution is filtered and dried under vacuum until all the acetic acid is removed. This residue is dissolved in several cubic centimeters of ether and placed in a partly covered weighing bottle in the refrigerator for 2 weeks to crystallize. If crystallization does not occur in this time, the solution is evaporated and the residue, dissolved in 4 to 5 cc. of 50-percent acetic acid, is filtered and dried as before. This residue is now dissolved in 2 to 3 cc. of anhydrous ether and left in the refrigerator as before for 2 weeks. When crystallization has occurred the ether solution is decanted, the crystals are dissolved in cold anhydrous ether, and this solution is replaced for crystallization as before. By such successive crystallizations as this pure rotenone is obtained.

The determination of rotenone was discussed by Koolhaas and Meijer (79) in 1938. Not only was some rotenone left uncrystallized but varying amounts remained in the mother liquor. As an extreme case, that of a root with a total-extract content of 25 percent and an actual rotenone content of 3 percent, rotenone amounting to 2.2 percent might be left in the mother liquor. In a root of this type by the usual method only about 60 percent of the true rotenone content would be obtained.

Methods for determining rotenone were discussed at length by Bertaud-Rossi (6) in 1938. The method of Jones (62) was said to give satisfactory results, but several changes designed to give more rapid results were introduced. To obtain ready crystallization of the rotenone from carbon tetrachloride, the extract should be dry. Rather than dry the sample itself and thus cause decomposition of the rotenone, this investigator dried the carbon tetrachloride extract over anhydrous sodium sulfate. The time of crystallization varied with the proportion of rotenone to resin; crystallization took place rapidly in extracts rich in resin. A device for enclosing the filtering crucible in an ice bath to maintain the

filtration at 0° C. was described and illustrated. In case a rapid result was desired, instead of waiting for the carbon tetrachloride solvate to come to constant weight, it could be dissolved in ether, the solution evaporated, and the process repeated. This scheme sufficed to remove the carbon tetrachloride completely, and the dried product was weighed as rotenone.

Meijer (87) reported in 1938 that preliminary drying of the derris root sample at 60° and 80° C. caused a marked lowering in the rotenone results. Thus, a sample originally analyzing 10.6 percent of rotenone gave the following results after drying:

Temperature °C.	Time Hours	Rotenone Percent
40	2	10.3
60	2	8.1
80	0.5	6.2
80	2	5.2

The Imperial Institute (58) reported in 1938 on work done by the Department of Agriculture of the Federated Malay States. Soxhlet extraction with carbon tetrachloride had been abandoned, and extraction with cold chloroform had been adopted as a routine method for rotenone. Results by this method fell only a little short of those by extraction with hot chloroform and were in agreement with results obtained with hot ethylene dichloride and ethyl acetate.

Graham (44), in his 1937 report to the Association of Official Agricultural Chemists, described work on the determination of rotenone done in collaboration with Jones, and recommended that the chloroform extraction method of Jones and Graham (72) be adopted by the Association as a tentative method.

A titrimetric step in the procedure for determining rotenone, making use of the rotenone-dichloroacetic acid solvate, was described by Jones (67) in 1938. With dichloroacetic acid rotenone was found to form a solvate containing an equimolecular proportion of the two constituents. Formation of this solvate was adapted to determining the purity of the crude carbon tetrachloride solvate obtained in the usual gravimetric crystallization method. The method was briefly as follows:

> The carbon tetrachloride solvate obtained in the usual way is dissolved in acetone, and the solution is evaporated to remove carbon tetrachloride. The residue is dissolved by warming in 10 cc. of 80-percent dichloroacetic acid. The solution is cooled in an ice bath, 10 cc. of water is added slowly, and crystals of the dichloroacetic acid solvate are added for seeding. After the solution has been 2 or 3 minutes in the ice bath, water is added 10 to 15 drops at a time, with cooling in the ice bath between additions, until about 25 cc. has been added. Then 25 cc. more of water is added dropwise and finally, after further cooling, 50 cc. of water is added more rapidly. After some additional cooling the crystals are filtered on a Gooch and washed with about 250 cc. of

water. The crystals are then dissolved in chloroform, about 50 cc. of water is added, and the mixture titrated with 0.1 N alkali using phenolphthalein as indicator.

With proper care in the addition of water during the precipitation, the acid solvate separated in a crystalline form from which excess acid was readily washed. In tests on specially prepared carbon tetrachloride solvates, the method gave values for purity about 2 percent higher than those by the older alcohol-recovery test. Results on samples of powdered root were in good agreement with those by the older gravimetric procedure. The procedure effected a saving in time over the gravimetric method and neutral impurities, such as sulfur, did not interfere. Attempts to precipitate the acid solvate directly from whole derris and cube extracts in an effort to shorten the procedure still further met with failure.

In his 1938 report to the Association of Official Agricultural Chemists, Graham (47) gave the results of a collaborative study comparing the crystallization method of Jones and Graham (72) with the titration method (67). The former method gave good results on both derris and cube, but several collaborators had difficulty with the latter procedure.

At the 1938 meeting of the National Association of Insecticide and Disinfectant Manufacturers, Graham (45) reviewed methods of analysis for rotenone. Extraction of the sample with carbon tetrachloride in a Soxhlet, although widely used, was said to have several disadvantages, among which were difficulty in obtaining complete extraction and decomposition of the solvent due to moisture present, with consequent decomposition of the rotenone. Chloroform extraction at room temperature was recommended. It was stated that a modification of the crystallization method had been developed for the determination of rotenone in the presence of sulfur. The method was not described, but it was said that the rotenone and contaminating sulfur were weighed and a correction was then made for the amount of sulfur present. The titration method was also said to be applicable to such mixtures.

Details of the method used in the presence of sulfur have been furnished by the Agricultural Marketing Service (126). Extraction was carried out in the usual way and the evaporated extract was then treated as follows:

> "Add 30 cc. of acetone to the residue, warm to dissolve the rotenone, cool in running tap water for 15 minutes, filter through a disc of filter paper in a Gooch crucible and wash 2 or 3 times with 10 cc. portions of acetone. Transfer the acetone solution to a 125 cc. Erlenmeyer flask and evaporate almost to dryness on a steam bath in a current of air. Then completely remove the remainder of the solvent under reduced pressure."

The remainder of the procedure was the same as that usually employed, beginning with the addition of carbon tetrachloride.

In 1939 the Imperial Institute (59) reported the results of work carried out in collaboration with the Rothamsted Experimental Station. Throughout the investigation three samples of derris were used, one each of *D. malaccensis* (Kinta (Sumatra) type), *D. malaccensis* var. *sarawakensis*, and *D. elliptica*, Changi (high rotenone). The method used by the Imperial Institute (I) involved percolation of the root with cold ethyl acetate, crystallization from carbon tetrachloride, and purification of the solvate by trituration, if necessary twice, with alcohol. In the Rothamsted method (II) the root was extracted in an automatic percolator with boiling ethyl acetate, chloroform, or ethylene dichloride. The dried extract was dissolved in ether and this solution extracted with dilute alkali to remove toxicarol and other alkali-soluble materials. The alkali-insoluble portion was subjected to crystallization from carbon tetrachloride, and the rotenone was calculated from the weight of solvate. In method I it was found necessary to add rotenone to obtain separation from the Kinta type root. The general concordance by method II was good.

As a result of this work method I was modified so that extraction was made by hot percolation, pure rotenone was added if necessary, and a second crystallization of the solvate from carbon tetrachloride was introduced. Method II was modified by the use of an ether-benzene mixture (25 percent benzene) instead of ether as solvent during alkali extraction of the resin. In method I ethyl acetate, ethylene dichloride, and chloroform were tried; in method II only chloroform was used. Purity of the complex was determined in both cases by crystallization from absolute alcohol. The results by method I indicated that the three solvents tried were of about equal value as extractants, even for the high-rotenone root. It was also found that addition of rotenone to aid crystallization in the case of the high-rotenone root effected no improvement in yield. When method II, involving removal of alkali-soluble material, was used, it was unnecessary to add rotenone to produce complete crystallization even in the case of the Kinta type root. In general, results by both methods at both laboratories were in fair agreement, although values for the Kinta-type root showed considerable variation.

Because the purity test was unsatisfactory, further work was done on schemes for determining purity. In method I the first crystallization was made at two concentrations, namely, from a volume of carbon tetrachloride (a) numerically 10 times the weight of resin, and a volume (b) 10 times the weight of resin plus added rotenone. In each case the complex was recrystallized from carbon tetrachloride, and the purity of the second complex was determined both by recrystallization from alcohol and by optical rotation. In method II the first complex was crystallized in the usual way and recrystallized from carbon tetrachloride, and the purity of this complex was determined by optical rotation. Values by method II were in good agreement. In method I there was no appreciable difference in the purity of second solvates obtained from solvates originally crystallized at dilutions (a) and (b). In general, the purity of the second complex as determined by rotation agreed with the value obtained by alcohol recrystallization when the determinations were made in either laboratory. In method I, however, when dilution (a) was used, there were serious discrepancies between the values obtained by the two laboratories. For this reason additional work was done at the Imperial

Institute by a worker from each laboratory, using both general methods and the several purification schemes. There was good agreement between values obtained by the two workers. Agreement between methods I and II was also generally good. It was pointed out that earlier discrepancies might have been due to the fact that at one laboratory the carbon tetrachloride used for crystallization was prepared by dissolving the theoretical weight of rotenone in the solvent while at the other an excess of crystals was kept in the solvent in the refrigerator. The former method was now agreed to be the better.

Some analyses were made by the two general methods and using titration to determine purity (67). In method I abnormally high results were obtained on the Kinta-type root, but in method II, after removal of alkali-soluble material, this root gave values that agreed with previous figures by other methods of measuring purity. The titration method appeared to be applicable only to solvates of a certain degree of purity.

In 1939 Cahn and Boam (16) confirmed the finding of Jones that the dichloroacetic acid and alcohol-recovery procedures gave substantially the same results. The relation between rotenone content and values by the Goodhue (40) color test was studied further. There was found to be an approximate relation between the values by this test and the pure-rotenone content, but the correlation was more exact when crude-rotenone content was considered. An abnormally small difference between Goodhue value and rotenone content was said to indicate the presence of Sumatra-type root or extract.

In 1939 Graham (46) reported that, in the analysis of cube powders by the chloroform-extraction method, higher percentages of rotenone were obtained, and the rotenone-carbon tetrachloride solvate crystallized more readily and was purer when decolorizing carbon was used in the extraction flask. When 10 gm. (Norit-A) was mixed with 30 gm. of cube powder and extracted in the usual way, the values for rotenone were from 0.5 to 0.8 percent higher than when no carbon was used. The use of carbon with the derris tested caused no significant difference in the results for rotenone.

Braak (9) in 1939 reviewed the results of analysis of the sample of derris root submitted by the Dutch East Indies authorities to various laboratories throughout the world and which previously had been reported by Koolhaas and Meijer (see p. 26). Braak also gave the results obtained on another sample of derris prepared in the Laboratory for Chemical Research at Buitenzorg, Java, and submitted to the laboratories of Seil, Putt, and Rusby in New York and of Salamon and Seaber in London. The sample, with approximately 8.5 percent of rotenone and 22 percent of total extract, was analyzed by the methods of Seil (see p. 25) using longer extraction time, Jones and Graham (72), Seaber (111), and Koolhaas (78). The Laboratory for Chemical Research and Seil, Putt, and Rusby, when applying the method of Jones and Graham, obtained practically identical results both for pure and crude rotenone. The Laboratory for Chemical Research found that the values for crude rotenone by the Jones and Graham method were almost the same as those for pure rotenone by the method of Koolhaas. The method of Seaber gave very different results when applied in the Dutch East Indies and in the commercial laboratory in London.

The method of Seil gave results considerably lower than by the other methods. However, another sample prepared by the Dutch laboratory and analyzed by Seil, Putt, and Rusby gave slightly higher results by the Seil method than by the Jones and Graham method. Although Braak believed the method of Koolhaas to give figures closer to the actual rotenone content, he stated that it would be most desirable for the method of Jones and Graham to be applied universally, since it satisfied all reasonable demands for reproducibility in various laboratories and the chance of its general acceptance seemed better than for any other method.

Bégué (5) in 1939 stated that the technique of Worsley was one of the best gravimetric procedures for rotenone determination, and he advised its use for derris root.

Numerous methods for evaluating rotenone-bearing plants were critically reviewed by Guillaume and Hervé (53) in 1939. All crystallization methods were said to be impractical for determining small quantities of rotenone, as crystallization is not effected. Increasing the size of the sample was said to diminish the sensitivity of the method. Such methods were said to be inapplicable to leaves and fruits. Colorimetric methods or a determination of methoxyl groups was recommended.

Georgi and Teik (37) in 1939 stated that the findings of Seaber (111) on decomposition of rotenone after prolonged boiling with carbon tetrachloride had been confirmed in their laboratory. Consequently they had discontinued the use of this solvent and substituted room-temperature extraction with chloroform. Extraction and crystallization were carried on as in the methods of Beach (3) and Jones and Graham (72), except that the extract was allowed to crystallize for 2 days. One gram of pure rotenone was added to extracts of roots containing 6 percent or less of rotenone. After separation of the first crop of crude carbon tetrachloride solvate, the volume of the mother liquor was reduced to 15 cc. and placed in the refrigerator for 1 day to obtain a second crop of crystals. They were added to the first crop and the purity of the whole was determined by the alcohol-recovery method of Cahn and Boam (13).

Graham (49) in his 1939 report to the Association of Official Agricultural Chemists, described the results of collaborative analyses of five samples of derris, timbo, and barbasco by the crystallization method (72) and by the titration method (67). Decolorizing carbon was used in the extraction flask as a result of the findings of Graham (46) on this point. Results were in fairly close agreement except on one sample of derris root of low rotenone content, which gave poor results by both methods. It was recommended that the crystallization method, adopted as "tentative" in 1937, be amended to include the use of decolorizing carbon and be adopted as "official, first action."

Graham (48) in 1940 reviewed the results of the collaborative analyses just described. He pointed out that the addition of decolorizing carbon resulted in higher values for cube samples, and stressed the necessity of using the multiple-extraction procedure with chloroform for samples with a ratio of rotenone to total extract greater than 40 percent.

In 1940 Meijer and Koolhaas (89) described the method in use at the Laboratory for Chemical Research, Buitenzorg, Java, for determining rotenone in derris root. This is a modification of the original method of Koolhaas (78), and since it is one of the more important methods now in use it is presented in some detail here:

"Extraction. Fifty grams of powder (at least 75 percent must pass an 80 mesh sieve) are percolated with ether in a Soxhlet, without a thimble but with cotton wool at the bottom, for 65 hours. The heat used for boiling the ether comes from a 60-watt electric bulb.

"Distilling off the Solvent. The ether is distilled off in a 100 cc. centrifuge tube on a water bath; the tube is filled with the ether solution by means of a dropping funnel. The rotenone which may have separated during the extraction is transferred quantitatively to the tube, and the flask is rinsed out with ether a few times. The final solution in the tube must be 25 cc. The tube is then tightly closed with a cork.

"Crystallization of Rotenone. The tube with the ether extract is kept at room temperature for 1 day and then in a refrigerator for 2 days. The mother liquor is poured into a 50 cc. Erlenmeyer flask and the remaining crude rotenone is broken up with a spatula, with the addition of 10 to 15 cc. of ether. The tube and the Erlenmeyer flask are closed with a cork and placed in the refrigerator for another day.

"Determination of Rotenone. The rotenone is centrifuged for 3 to 5 minutes at 3500 revolutions per minute and the supernatant liquor is added to the mother liquor in the Erlenmeyer flask. The centrifuge tube with the crude rotenone is dried for 10 minutes in a water bath at 70° C., and after a slow current of air has been passed into the tube, it is dried in vacuo on a boiling water bath for 15 minutes. After cooling in a desiccator the tube is weighed. * * * The purity of the crude rotenone is determined by the melting point, using an empirical table in which the correlation between the purity and melting point is given. If the melting point is lower than 140° C., the mass in the centrifuge tube is treated with another 10 cc. of ether, centrifuged, and dried, and the melting point is again determined * * *. The authors determine the optical rotation, from which the purity of rotenone can also be determined. A correction is made for the amount of rotenone dissolved in the ether of the mother liquor and the ether used for washing. For each cubic centimeter of ether used 4.2 mg. of rotenone are added to the amount of pure rotenone. If rotenone has separated from the mother liquor to which the wash ether has been added, after standing in the refrigerator for another night, it is centrifuged off and added to the crude rotenone * * *.

These authors attempted to determine by two schemes the amount of rotenone left in the resin after crystallization. The first involved extraction of the nonrotenone resins with petroleum ether and cyclohexane and crystallization from carbon tetrachloride of the rotenone in the residue, while the second method was based on adsorption on activated fuller's earth and washing out with benzene, the evaporated residue being crystallized from carbon tetrachloride. From a number of samples of resin a range of from 4.5 to 17.0 percent (with two exceptions), with an average of about 10 percent, of the amount of the original rotenone was recovered.

Results of the analyses of 40 samples of derris root by the authors' method and by that of Jones and Graham (72) were compared. The results for pure rotenone by the authors' method were in close agreement with those for crude rotenone by the latter method. It was found that finer grinding in general resulted in higher values for both rotenone and total ether extract. Meijer and Koolhaas believed that samples with a ratio of rotenone to total extract higher than 40 percent, which were said to be common in the Dutch East Indies, could be handled satisfactorily either by finer grinding (75 percent through a 200-mesh sieve) or by extraction several times with chloroform. They stated that a satisfactory uniform method for rotenone might be based on the Jones and Graham method, provided the values for crude rotenone were used and this method was adapted to samples with a high ratio of rotenone to total extract. The authors compared results for purity of the carbon tetrachloride solvate by polarization, alcohol recovery, and the titration method of Jones (67). The results were generally in good agreement; alcohol recovery in general gave the lowest values, polarization highest, and titration between these two. The degree of purity when determined by the melting point was said to be generally higher than when determined either by polarization or alcohol recovery. As they experienced difficulty with the titration method when 20 percent or more impurity was present and as the polarization method was even less time-consuming, the authors preferred the latter method. The figures previously given by Meijer (87) to show the effect on the rotenone content of drying the sample were repeated. Heating the sample above 50° C. before analysis was strongly discouraged.

In further work on the evaluation of rotenone-containing plants, Martin (83) in 1940 described the procedure for determining rotenone in derris, which involved some modification of previous methods. Sufficient root to give about 5 gm. of resin was extracted by hot percolation with ethyl acetate for 3 hours in the apparatus already described under methods for Total Extract. The resin, freed of solvent, was dissolved in 100 ml. of a mixture of benzene and ether (25 percent by volume of benzene) and extracted rapidly with 50 ml. of 2-percent potassium hydroxide and then with two lots of 5-percent potassium hydroxide. Water was then immediately added to the benzene-ether solution to dilute any residual alkali. The combined alkaline extract was washed with benzene-ether and the wash added to the main solution. The combined benzene-ether solution was then washed three times with water and dried with anhydrous sodium sulfate, and the solvent was removed. The resin was dissolved in carbon tetrachloride and the rotenone solvate crystallized in the usual way. Determination of the

purity of the complex was carried out by a polarimetric method. The optical rotation of a 4-percent solution of the solvate in benzene was determined, and the percentage of rotenone then calculated from the rotation of pure carbon tetrachloride solvate in benzene. It was stated that impurities present were likely to show a specific rotation approximately one-fourth that of rotenone. The error involved was said to be small but might be allowed for if it was considered necessary. Values obtained by this method, in general, agreed with those obtained by the dichloroacetic acid titration method (67).

The Imperial Institute (57), in a survey of insecticides from plant materials in 1940, briefly discussed the evaluation of derris and cube. It was stated that results for rotenone depended on the solvent, the number of extractions, the temperature and length of time of extraction, as well as on the fineness of grinding and moisture content of the sample. The need of a standardized method was stressed.

In the Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists (2) the following method for determining rotenone, that recommended by Graham (49), is given as "official, first action":

"Weigh 30 g. (if sample contains more than 7% rotenone use a quantity that will give 1.0 - 1.5 g. of rotenone in the 200 ml. aliquot) of finely powdered root and 10 g. of decolorizing carbon into 500 ml. glass-stoppered Erlenmeyer flask. Add 300 ml. of chloroform measured at definite room temperature; place flask on shaking machine and fasten stopper securely. Agitate vigorously for not less than four hours, preferably interrupting shaking with overnight rest (or flask may be shaken continuously overnight). Remove flask from machine and allow to cool in refrigerator for at least an hour. Filter mixture rapidly into suitable flask, using fluted paper without suction and keeping funnel covered with watch glass to avoid loss from evaporation. Stopper flask and adjust temperature of filtrate to that of original chloroform.

"Transfer exactly 200 ml. of this solution to 500 ml. Pyrex Erlenmeyer flask and distil until only about 25 ml. remains in flask. Transfer extract to 125 ml. Erlenmeyer flask, using carbon tetrachloride to rinse out the 500 ml. flask. Evaporate almost to dryness on steam bath in current of air. Then remove remainder of solvent under reduced pressure, heating cautiously on steam bath when necessary to hasten evaporation (suction may be applied directly to flask). Dissolve extract in 15 ml. of hot carbon tetrachloride and again, in a similar manner, remove all solvent. Repeat with another 10 - 15 ml. portion of hot carbon tetrachloride. (This treatment removes all chloroform from the resins. The chloroform extract is usually completely soluble in carbon tetrachloride. If small quantities of insoluble material are present, the purification procedure described later will eliminate them. However, if large quantity of insoluble residue should remain when extract is dissolved in first portion of carbon tetrachloride, it should be filtered off and thoroughly

washed with further portions of hot solvent, after which the filtered solution plus washings should be treated as directed above for removal of chloroform.)

"Add exactly 25 ml. of carbon tetrachloride and heat gently to completely dissolve extract. Cool flask in ice bath for several minutes and seed with a few crystals of rotenone-carbon tetrachloride solvate if necessary. Stopper flask and swirl until crystallization is apparent. If at this stage only a small quantity of crystalline material separates, add an accurately weighed quantity of pure rotenone estimated to be sufficient to assure that final result, expressed as pure rotenone, is at least 1 g. Then warm to effect complete solution, and again induce crystallization. At same time prepare a saturated solution of rotenone in carbon tetrachloride for washing. Place flasks containing extract and washing solution in ice bath capable of maintaining temperature of 0° C. and allow to remain overnight.

"After 17-18 hours in ice bath, rapidly filter extract through weighed Gooch crucible fitted with disc of filter paper, removing flask from ice bath only long enough to pour each fraction of extract into crucible. Rinse residue of crystalline material from flask and wash under suction with sufficient of ice-cold saturated solution (usually 10 - 12 ml.) to remove excess mother liquor. Allow crucible to remain under suction for about 5 minutes and then dry to constant weight at 40° C. (about an hour). The weight obtained is 'crude rotenone-carbon tetrachloride solvate'.

"Break up contents of crucible with spatula, mix thoroughly, and weigh 1 g. into 50 ml. Erlenmeyer flask. Add 10 ml. of alcohol that has previously been saturated with rotenone at room temperature, swirl flask a few minutes, stopper tightly, and set aside at least four hours, preferably overnight, at the same temperature. Filter on weighed Gooch crucible fitted with disc of filter paper. Rinse crystals from flask and wash under suction with solution of ethyl alcohol saturated with rotenone at temperature of recrystallization (ca. 10 ml will usually be required). Allow crucible to remain under suction 3 to 5 minutes and then dry at 105° C. to constant weight, which should be effected in 1 hour.

"Multiply weight expressed in grams by weight of the 'crude rotenone-carbon tetrachloride solvate' and to product add 0.07 g. which represents correction for rotenone held in solution in the 25 ml. of carbon tetrachloride used in crystallization. If any pure rotenone has been added, subtract its weight from value obtained. This gives the weight of pure rotenone contained in the aliquot of the extract, representing 20 grams of the sample.

"Alternate extraction procedure. - If sample is one in which ratio of rotenone to total extract is greater than 40%, use quantity sufficient to contain 1.0 - 1.5 g. of rotenone and successively extract four times with $CHCl_3$, using 200 ml. each for second to fourth extractions. Filter after each extraction and return marc

to flask for extraction with fresh solvent. Finally combine extracts, evaporate almost to dryness, and proceed as directed above, beginning at point where aliquot has been evaporated almost to dryness."

The same commercial sources that supplied information on the determination of total extract also discussed the methods used by them for the determination of rotenone (private communications). Two of them, McCormick and Company and John Powell and Company, employed the official A. O. A. C. method. The laboratory of S. B. Penick and Company, regularly used the official method but also occasionally employed a modification for comparative purposes. This method, which depended on measuring the carbon tetrachloride of crystallization and assumed that rotenone is the only solvated material present, was as follows:

Place the crucible containing the crude solvate (dried to constant weight) in a 100-cc. beaker and weigh. Add 5 cc. of alcohol (or acetone) to the material in the crucible and keep in a moderately warm place, where evaporation will proceed slowly, until all the solvent has evaporated. Repeat this step twice and then dry the beaker and contents at 100° C. to constant weight. This procedure removes the carbon tetrachloride of crystallization, which was combined in equimolecular ratio with the rotenone in the solvate. The difference in weight divided by 0.281 and multiplied by 0.719 gives the weight of rotenone.

Another firm, Derris, Incorporated, preferred to use acetone as the extraction solvent, claiming that this solvent extracted the resins and rotenone in less time than any other solvent tried. The method was briefly as follows:

From 40 to 50 gm. of the coarsely ground or 25 to 50 gm. of finely powdered root is extracted in a Soxhlet with acetone. (If powdered root is used, it is mixed with an equal volume of fine sand or sodium chloride.) At the end of 3 hours the extraction flask is replaced by another containing fresh acetone and the extraction continued for about 20 minutes, or until a negative color test (Durham) shows that the marc is depleted of rotenone. The combined acetone extracts are then concentrated to about 10 cc., when two or three additions and evaporations of 25 cc. each of carbon tetrachloride are made to remove the acetone.

One hundred cubic centimeters of carbon tetrachloride and about 3 gm. of Celite are added to the residue and the whole is heated to boiling and filtered by suction. The residue is washed with two or three lots of about 25 cc. each of hot carbon tetrachloride. The carbon tetrachloride solution is concentrated to about 25 cc. and transferred to a 50-cc. graduated cylinder with ground-glass stopper. The volume is adjusted to 40 cc. with carbon tetrachloride and the cylinder placed in the refrigerator to remain overnight. The filtration, washing, and determination of purity are carried out approximately as in the official A. O. A. C. method.

Results by this method were said to be in agreement with those by the various published procedures, including the official A. O. A. C. method.

Discussion

It is generally agreed that derris and cube root should be finely ground and air-dried before extraction. Several investigators (71, 87) have shown that drying at elevated temperature is detrimental to the determination of rotenone.

One of the best solvents for extracting rotenone for analytical purposes is chloroform. Extraction at room temperature seems to be preferred by most workers. This method is rapid, yet it extracts less extraneous material and involves less decomposition than the use of other solvents or higher temperatures. For most roots the aliquot procedure appears to be entirely suitable, but for roots with a high proportion of rotenone, as pointed out by Jones and Graham (71) and Meijer and Koolhaas (89), a more exhaustive extraction must be resorted to. For this reason the alternative multiple-extraction procedure is included in the official A. O. A. C. method (2).[8]

Crystallization from carbon tetrachloride as carried out by most of the present methods may be considered to be reasonably complete. It is possible, as indicated by the work of Cahn, Phipers, and Boam (17), and of Meijer and Koolhaas (89), that in some few samples, particularly those with a very low ratio of rotenone to total extract, a small proportion of the rotenone remains unaccounted for in the mother liquor. While the reviewer (66) believes this to be primarily a result of greatly retarded rate of crystallization in certain types of extracts, other investigators (17) state that it is due to an actual solvent effect of other constituents. Whichever may be the case, anything that can be done to increase the relative proportion of rotenone will aid in producing more nearly complete crystallization in the time allowed. The addition of pure rotenone (13, 66), the treatment with decolorizing carbon (130, 46), and the removal of alkali-soluble material (84) all tend to accomplish this result. In the reviewer's opinion, when these schemes or combinations of them are employed, samples in which rotenone remains unaccounted for in the resin will be encountered only rarely, and even then results will not be greatly in error. If derris samples with a very low proportion of rotenone to total extract are to be encountered regularly on the market, alkali extraction may become a necessary step. Such roots as these usually contain a large proportion of toxicarol and other phenols, and their removal undoubtedly improves the crystallization of the rotenone.

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[8] Recently a sample of derris root encountered in the laboratories of this Bureau was not completely extracted even by this method and it was necessary to resort to hot extraction. This sample contained about 10 percent of rotenone and 24 percent of total extract.

Most investigators now feel that pure rather than crude rotenone should be reported. With regard to the best method of determining purity there is some difference of opinion. The alcohol-recovery method (13, 63) is the most used, although it generally gives slightly lower values than other methods. The titration scheme (67) may give more accurate values, but difficulty has been experienced in applying it to low-rotenone roots. Polarization (111, 89, 130) is used but probably gives values that are slightly too high. According to Meijer and Koolhaas (89) the purity determined by melting point is generally higher than by polarization. In any of these methods more nearly the correct value will be obtained the purer the original solvate. Since the three methods already discussed for treating the extract before crystallization — namely, addition of rotenone, use of carbon, and alkali extraction — in general result in a purer solvate, they are to be recommended from this standpoint as well as from that of improving the completeness of crystallization. Thus it appears from work reported by the Imperial Institute (59) that alkali extraction might obviate much of the difficulty encountered by some workers in the use of the titration method with low-rotenone roots.

Without doubt the procedure for the determination of rotenone that involves room-temperature extraction with chloroform and crystallization from carbon tetrachloride, as exemplified by the official A. O. A. C. method (2), is now the most widely used.

DEGUELIN AND ROTENONE PLUS DEGUELIN

The Durham Test

A test widely used in work on rotenone-containing plants is that based on the color reaction discovered by Durham in 1902, in which treatment of certai constituents of derris root with nitric acid followed by ammonia was found to produce an evanescent blue-green color. Ishikawa (60) in 1916 independently discovered this reaction, using sodium hydroxide as the base. According to Tattersfield and Roach (122) and Gimlette (38), who described Durham's work, concentrated nitric acid was added to solid rotenone, or resin, and this mixture was treated directly with strong ammonium hydroxide. In this form the test was qualitative only and was unsuitable for delicate testing because of the violence of the neutralization.

Peyer and Hünerbein (92) in 1931 described a qualitative test similar to that of Durham. To 2 cc. of dilute acetic acid extract of the sample, 2 drops of fuming nitric acid were added; the mixture was diluted with 10 cc. of water and then made alkaline with sodium hydroxide. The color produced passed quickly from green to brown. The green color produced with ammonia was said to be more stable than that obtained with sodium hydroxide.

In 1933 Jones and Smith (75) modified the Durham test in an attempt to render the blue color more permanent and make the test more nearly quantitative. They used the following procedure:

> To 1 cc. of an acetone solution of rotenone (or a plant extract) 1 cc. of 1 + 1 nitric acid is added, and the mixture is allowed to stand for 1/2 minute. It is then diluted with 8 to 9 cc. of water and 1 cc. of strong ammonium hydroxide is added. A blue color is produced which is almost identical with that given by bromothymol blue indicator at a pH of 7.2.

It was said that as little as 0.1 mg. of rotenone could be detected by this method. By preparing a series of solutions containing different concentrations of bromothymol blue in a buffer of pH 7.2 and standardizing these against the colors produced by different amounts of rotenone, it was possible to use the test to make a rough estimate of the amount of rotenone present. Since approximately the same intensity of color was given by deguelin, whereas toxicarol gave almost no color, it was believed that the test gave a rough value for the total rotenone plus deguelin content of plant extractives. Sodium and potassium hydroxides, or sodium and potassium carbonates, used in place of the ammonia, were also found to produce the blue color. When the nitric acid was partially neutralized with sodium bicarbonate and the neutralization was completed with a stronger base. This color was slightly more permanent.

The first reported semiquantitative application of this test was made by Jones, Campbell, and Sullivan (70) in 1935, in working with a series of samples of several species of *Tephrosia*. Extracts that gave the test were rated in three grades depending on the depth of color produced.

Fischer and Nitsche (23) in 1935 made a study of the tests proposed by Peyer and Hünerbein (92) and by Jones and Smith (75) in a further attempt to render the reaction more nearly quantitative. The test, carried out in

essentially the same manner as already described except that the diluted reaction mixture was cooled to 0° C. before addition of the ammonia, was as follows:

> To prepare color standards, 50 mg. of malachite green is dissolved in 1 liter of water, a portion of the solution is diluted to double the volume and the procedure is repeated until six solutions of successively greater dilution have been prepared. In general, in the analysis of rotenone and rotenone-rich extracts a 0.4-percent solution in acetone is needed. One part is diluted to double the volume and another to four times the volume, so that the three solutions contain 4, 2, and 1 mg. per cubic centimeter of rotenone or extract. At the time of analysis 1 cc. of acetone solution is mixed with 2 cc. of dilute nitric acid (sp. gr. 1.2), allowed to stand 2 minutes, diluted with 7 cc. of ice-cold water, and cooled to 0° . Two cubic centimeters of 25-percent ammonia is then added rapidly, and the liquid is quickly mixed and compared visually with the standards. Estimations must be made not later than 3 to 4 seconds after addition of the ammonia. Orange-yellow color filters may be used in making the comparison. For a repetition of the measurements another series of concentrations of the unknown sample should be chosen. Values obtained may be plotted graphically with the standards if desired. As the end value of an analysis the middle value from three observations at different concentrations may be used. It should not differ more than about 10 percent from the end value of a second analysis.

In some cases extraction with benzene followed by evaporation and solution in acetone removed impurities that interfered with the measurement of the color. In the presence of many substances the appearance of the yellow color with nitric acid was very much retarded, and in these cases it was necessary to add a drop of fuming nitric acid to initiate the first reaction.

The colorimetric method of Jones and Smith (75) was regarded by Goudswaard and Timmers (43) in 1937 as unsuitable for the estimation of rotenone, because the intensity of the color produced depended on the temperature and because various tints interfered with the estimation of the color.

Pozzi-Escot (98) in 1937 stated that bases other than ammonia produced the final blue color in the Durham reaction. Even organic bases such as triethanolamine produced a blue-green color.

Sievers and coworkers (113) in 1938 extended the semiquantitative use of the Jones and Smith (75) variation of the Durham reaction to several hundred samples of Tephrosia virginiana. Extracts were rated in five different grades depending on the degree of color, and two investigators working independently gave substantially the same rating to the majority of samples.

The Durham reaction has been adapted to the qualitative testing of mineral-oil fly sprays for rotenone and deguelin (125). Directions for the test were as follows:

"Transfer 5 cc. of the material to a large test tube, add 2 cc. of concentrated nitric acid and shake for 20 to 30 seconds, dilute immediately with 20 cc. of water. Close the tube and shake to disperse the oil in the aqueous solution; allow the oil to separate, which should not require more than 30 to 40 seconds, and add 1 or 2 cc. of ammonia poured down the side of the tube. The presence of rotenone or derris extract is indicated by the formation of a fugitive blue color."

Quantities of rotenone as small as 2.5 mg. in 5 cc. of a mineral oil-pyrethrum extract were said to have been detected by this method.

Guillaume and Hervé (53) in 1939 described a modification of the Durham test which rendered the color slightly more permanent and permitted a roughly quantitative estimation. This scheme involved adding only a few drops of nitric acid to the acetone solution of rotenone or extract, covering with a layer of toluene and allowing to stand in vacuo at -8° to -10° for 1 hour. The blue color then obtained by adding a few drops of ammonia persisted unchanged for about 1 minute. Colors produced simultaneously from the sample and from standard solutions of rotenone were compared rapidly in test tubes. In this form the method was said to be sufficiently precise and sensitive for many purposes.

The Gross and Smith Test

A more useful color reaction than that of Durham from the quantitative standpoint was discovered in 1934 by Gross and Smith (50). It involved treatment of an acetone extract of the sample with alcoholic alkali followed by nitric acid containing nitrite. A rather permanent red color was given by both rotenone and deguelin, and also by dihydrorotenone. Briefly the procedure was as follows:

To 2 cc. of an acetone solution or extract containing 0.05 to 0.30 mg. of rotenone per cubic centimeter, add 2 cc. of 10-percent alcoholic potassium hydroxide solution, and allow to stand at 20° C. for 2 minutes. Then add 6 cc. of a nitric acid-sodium nitrite mixture containing 1 volume of concentrated nitric acid to 1 volume of aqueous sodium nitrite having 0.25 gm. of sodium nitrite per liter. Mix, cool to 20° C., and allow to stand at this temperature for 15 minutes. Compare visually with standards containing pure rotenone prepared at the same time.

When applied to samples containing only rotenone, the results agreed with those by the gravimetric methods. Applied to derris and cube samples, the results were 50 to 100 percent higher than for rotenone alone, owing to the presence of deguelin. The method was applied to rotenone spray residues on fruits and foliage.

In using the Gross and Smith test for total rotenone and deguelin in several samples of derris root, Tattersfield and Martin (120) employed two methods of extraction. In one method the samples were extracted with acetone and aliquots of this extract were diluted to the proper concentration for the

test. In the other procedure the samples were extracted with ether and the dried extract was dissolved in acetone and treated as in the first method. Colors developed from extracts by the second method were more readily matched than those obtained from the first method.

Ambrose and Haag (1) in 1936 shortened this test for use in detecting rotenone and deguelin in the excreta of animals fed rotenone and derris. The material was extracted with ether and the evaporated extract taken up in acetone. To this solution was added one-third its volume of freshly prepared 10-percent alcoholic potassium hydroxide, and the resulting mixture warmed. A wine-red color developed to a maximum in about 15 minutes. The test was said to be sensitive to 1 part of rotenone in 35,000.

Goodhue (40) in 1936 described a more serviceable modification of the Gross and Smith test, which has been widely used in subsequent work. Sulfuric acid was substituted for nitric, the concentration of the alcoholic potassium hydroxide was reduced, and the nitrite necessary to produce the color was added in the alkali instead of in the acid. The method was as follows:

> Prepare the following reagents: (a) Mix 1 volume of sulfuric acid (sp. gr. 1.84, free from nitrous acid) with 3 volumes of water. (b) Dissolve 1 gram of sodium nitrite in 10 cc. of water and dilute to 1 liter with 95-percent alcohol. (c) Dissolve 40 grams of potassium hydroxide in 100 cc. of water. (d) Mix 1 volume of reagent (c) and 7 volumes of reagent (b). Prepare this solution fresh daily.
>
> Proceed as follows: Prepare an acetone extract of the sample containing from 0.005 to 0.25 mg. of rotenone per cubic centimeter and pipette 2 cc. into a dry test tube. Add 2 cc. of reagent (d) and place the tube in a water bath at about 25° C. for 5 minutes. Add 5 cc. of reagent (a), stopper, shake, and place the tube back in the water bath. The color reaches a maximum after about 15 minutes, and then remains unchanged for 2 hours. Determine the amount of rotenone by comparing the color with standards prepared at the same time from known quantities of rotenone.

The turbidity or brown color which sometimes developed during the analysis of crude plant extracts was removed upon extraction of the final mixture with a small portion of ether. The red color due to rotenone and deguelin was not removed. In this work comparisons were made with Lovibond color slides in a roulette comparator. The small amount of blue which accompanied the red was filtered out by a dichromate filter for easier matching. By this modified procedure the sensitivity of the original test was increased 20 times. The specificity for rotenone and deguelin remained the same. Deguelin was said to give the same amount of color as did rotenone.

This method was criticized by Goudswaard and Timmers (43) in 1937 on the same grounds as the Jones and Smith (75) test-namely, dependence of color intensity on the temperature and interference of various tints with the estimation

of the color. On the other hand, the method was said by Schonberg (110) to present several advantages over the crystallization method. Among these were the fact that it would determine very small quantities and that it was rapid and practical. Furthermore, the oxidation of rotenone to dehydrorotenone could be followed by this colorimetric method but not by crystallization methods.

Cahn, Phipers, and Boam (17) in 1938, in an extensive study of the composition of derris root, used this colorimetric method, making color comparisons in a Duboscq colorimeter. They proposed to call the result obtained the "Goodhue value"; this was the percentage of rotenone that the material would contain if rotenone were the only ingredient giving a color in the test. They found these values to bear a definite relation to the rotenone content of derris extract. For ordinary extracts the relation was as follows: Goodhue value = percent rotenone + 22 ± 3. This relation did not hold for Sumatra-type extracts, which usually had Goodhue values of 10 to 15. As mentioned in the section on Rotenone, this relation was used by Cahn, Phipers, and Boam to determine approximate rotenone contents by the color method. According to these writers, Rotenone and deguelin were the only substances (known at that time) in derris extract which gave this color test. They found that the color given by deguelin was only four-fifths the intensity of that given by rotenone. Consequently, they stated that the "excess" value above the actual rotenone content must be multiplied by 1.25 to indicate the deguelin content of an extract. Using this value, they concluded the deguelin content of derris extracts to be 27 ± 4 percent, except Sumatra-type extracts, which contained 9 to 15 percent of deguelin.

Again in 1939 Cahn and Boam (16) discussed the determination of approximate rotenone content by means of the Goodhue value. This scheme has been mentioned in the section on Rotenone.

In a study of colorimetric procedures in 1939 Jones (68) used this method and made color comparisons in a neutral wedge photometer using a filter with its optical center at 0.56 micron. This means of measuring the color had been in use for some time by Goodhue and by Cassil in the laboratories of this Bureau.

Bégué (5) in 1939 recommended this method for the analysis of products containing less than 1 percent of active principles.

Guillaume and Hervé (53) in 1939 described and discussed Goodhue's modification of this method. They suggested that for this test a 1-gm. sample be extracted with 100 cc. of acetone in a Soxhlet or a Kumagava (81) apparatus for 5 to 8 hours. They also found that complete exhaustion of 1 gm. of powder was obtained by agitating with 100 cc. of acetone in a flask for 2 hours. They found it unnecessary to prepare a standard solution of rotenone each time the test was made. Instead, they made a 0.05-percent solution in acetone and kept it in the dark in ice (or at laboratory temperature) for use in a large number of determinations. The procedure used for the development of the color was essentially the same as that already described. Although this method gave results on freshly prepared powders agreeing with those of the blue color test and the methoxyl method, it gave lower results on old powders than the other two methods.

Goodhue and Haller (42) in 1940 used this method for the estimation of dihydrorotenone in the hydrogenation products of rotenone. Although this derivative of rotenone has not been found in derris and cube roots, the modified procedure is equally applicable to the determination of rotenone and deguelin in extracts of these plants. The procedure was practically identical with Goodhue's earlier modification (40) of the test except that 6 cc.(instead of 5 cc.) of dilute sulfuric acid was used following the addition of alkali and the solution was then maintained in a cold-water bath at 15 to 20° C. for about 5 minutes (instead of at 25° for a longer period). However, the measurement of the color intensity was improved. This portion of the procedure was as follows:

> "The use of a photometer has been found to be the most accurate method of comparing the standards and the unknowns. The colors can be developed directly in selected test tubes which fit the photometer, or they can be developed in any test tube and poured into a special cell. A Brice photometer which gives percentage transmission as direct readings has been found satisfactory. Glass filters (Corning, 3.5 mm., No. 430, dark shade blue-green) were used. The blank is taken as 100 percent transmission, and the photometer is therefore adjusted to give a reading of 100 with the cell containing the blank in place. The readings for the standards and unknowns which are next obtained are therefore in percent transmission.
>
> "***the color for rotenone has been found by Cassil (unpublished) to follow Beer's law***. A plot is therefore made on semilogarithmic paper with transmission as ordinate (logarithmic) and concentration as abscissa (arithmetic), and a straight line is drawn from the point of zero concentration and 100 percent transmission to the point determined by the concentration and the transmission of the standard. The transmission of the unknown having been determined, its concentration can be read from the curve and the percentage calculated."

Cassil (19) in 1941 used the red color test for the determination of derris-dust residues on cabbages. The residues were extracted by washing the leaves with chloroform, the extract evaporated, and the residue dissolved in hot acetone. The acetone solution was chilled to remove waxy material, and the filtered solution was used for the color test. The colors were compared with those from extracts of known quantities of the derris root actually used in the original dusts. Comparisons of color intensity were made in a photoelectric photometer.

Gravimetric Methods

Takei, Miyajima, and Ono (118) in 1933 developed a gravimetric method for determining rotenone and deguelin. This method, which has been briefly discussed under Rotenone, is based on the fact that rotenone and deguelin are readily oxidized under alkaline conditions to rotenolones and deguelinols, respectively, which are quantitatively dehydrated by alcoholic sulfuric acid to the highly insoluble dehydrorotenone and dehydrodeguelin. The mixture of dehydro compounds gives a value for total rotenone plus deguelin. A value for deguelin alone was obtained by subjecting the dehydro compounds to appropriate catalytic hydrogenation, which converted the dehydrorotenone to the alkali-soluble isodihydrodehydrorotenone (dehydrorotenonic acid) while the dehydrodeguelin remained unchanged. In the original method much of the rotenone was first crystallized out. The method is briefly as follows:

> The rotenone is first crystallized from an ether extract in the usual way. Five grams of the evaporated mother liquor is dissolved in 150 cc. of alcohol and 3 gm. of 5-percent alcoholic sodium hydroxide is added. Oxygen is passed in at the rate of 150 cc. per minute for 1/2 hour. If air is used instead of oxygen, it is run through for 1 1/2 to 2 hours. The reaction product is acidified with 15 gm. of 50-percent alcoholic sulfuric acid, and 130 cc. of alcohol is distilled off on the water bath during 1/2 hour. The residue is refluxed another hour. It is mixed with 500 cc. of water and shaken vigorously with 200 cc. of ether in a separatory funnel. The dehydro derivatives are suspended in the ether layer, other materials dissolve in either the water or the ether layer. The entire mixture is filtered by suction, and the crystals are washed with 10 cc. of methyl alcohol. The yellow needle crystals are dried at 100° and weighed. This value gives the weight of any rotenone not separated in the original ether crystallization plus the deguelin. A small quantity of crystals may also be obtained from the filtrate. Five-tenths gram of the mixture of dehydro compounds and 0.5 gm. of palladium-barium sulfate catalyst are placed in 100 cc. of alcohol and mixed with 3 cc. of 3-percent sodium hydroxide. The mixture is shaken in an atmosphere of hydrogen for 2 hours, and then filtered. The residue consists of dehydrodeguelin and catalyst. It is extracted with acetone, and evaporation of the extract gives the dehydrodeguelin. Most of the alcohol is distilled from the filtrate, which is then acidified with sulfuric acid and extracted with ether. Evaporation of the ether extract gives the isodihydrodehydrorotenone.

Danckwortt, Budde, and Baumgarten (25) in 1934 stated that the foregoing method might be theoretically unobjectionable but it was very troublesome and time-consuming and required a large amount of apparatus.

Fischer and Nitsche (28) stated that the method was too bothersome and time-consuming in its complete execution. They found it useful, however, for determining only the sum of rotenone and deguelin. Essentially the directions of Takai et al. were used, but in washing the separated dehydro compounds the methyl alcohol wash was followed with a small amount of ether. Frequently a

second or third methyl alcohol or ether rinse was necessary when the filtrate from the first was dark colored. In the case of plant materials inclined to resinify on oxidation, the substance was extracted with benzene and the evaporated benzene extract used for the oxidation. The disturbing materials were insoluble in benzene.

In 1935 Tattersfield and Martin (120) found that pure rotenone, when put through the first portion of the Takei process, gave a yield of only about 80 percent of dehydrorotenone. The remainder could be recovered from the solvents used for the final separation of dehydro compounds. Consequently they modified this separation. After dehydration with alcoholic sulfuric acid, the residue was cooled for some time in ice, the crystals filtered by suction and washed successively with a little ice-cold ether, 100 cc. of distilled water, and a few cc. of ice-cold methyl alcohol. The crystals were dried at 100° C. and weighed. The filtrate was then separated by adding 400 cc. of distilled water and 200 cc. of ether. If a further yield of crystals was obtained, they were filtered, washed with a little ice-cold methyl alcohol, and the weight added to that of the first lot. Tattersfield and Martin in some cases used the whole ether extract of derris without preliminary separation of the rotenone, and in other cases the mother liquor from the separation of the carbon tetrachloride complex, which they dissolved in the alcoholic mother liquor from the purification of the rotenone solvate. Higher results were obtained in general by the modified method, either with or without removal of the rotenone, than by the original Takei method made on the whole ether extract.

In the following year the same investigators (84) attempted the quantitative preparation of "deguelin concentrates" of several samples of derris root, following the qualitative scheme used in the earlier work of Haller and LaForge (55). Twenty grams of the roots was extracted with petroleum ether, with rapid refluxing, for 55 hours. The evaporated extracts were dissolved in ether, and the rotenone was allowed to crystallize for 2 days in the ice chest and then separated. The ether filtrates were then extracted with dilute potash, washed with water, dried over sodium sulfate, concentrated to a small volume, and placed in the ice chest for 5 days. Any further rotenone was separated. The mother liquor from this separation was termed the "deguelin concentrate." It probably contained most of the deguelin but may have contained other materials also. The values were somewhat higher than those obtained for dehydro compounds by the modified Takei method (120) on the alkali-insoluble portions of the original ether extracts. In this work also the term "rotenone plus deguelin" was applied to a value calculated from the methoxyl content of the alkali-insoluble portion of the extract and based on the methoxyl content of rotenone and deguelin of 15.74 percent.

In using Takei's dehydro method Worsley (132) in 1937 found the amount of wash solvents recommended by Tattersfield and Martin (120) to be inadequate. Accordingly he adopted the following procedure: The cooled residue from the dehydration process was filtered through a Gooch crucible and washed with 30 to 40 cc. of ether cooled to -10° C., the crystals were then pressed down and 100 cc. of water cooled to 2 or 3° C. was poured through; finally methyl alcohol cooled to -10° C. was poured through as long as any color was removed. The residue was dried and weighed. In some

cases the crystals were dark colored and resins appeared to be present. Hence the dried crystals were stirred with about 10 cc. of ether cooled to -10° C., filtered off, washed with 10 cc. more ether, then with 5 cc. of methyl alcohol cooled to -10° C. and finally dried and weighed again. The second series of washings gave purer crystals, and no further yield could be obtained from the filtrate.

Cahn, Phipers, and Boam (17) in 1938, in briefly discussing the method of Takei and coworkers, stated that they had found the reactions involved not to be quantitative for pure rotenone and deguelin, and that toxicarol behaved partly like deguelin and artificially swelled the resulting deguelin contents.

Jones (68) in 1939, in using this method, removed the alkali-soluble material prior to the oxidation to avoid interference from toxicarol. This plan had also been used in the work of Martin and Tattersfield (84).

In 1939 Goodhue and Haller (41) developed a method for determining deguelin in derris and cube based on its racemization and the separation of the inactive form as the carbon tetrachloride solvate. The method was as follows:

> A 50-gm sample is extracted with chloroform in a Soxhlet for 7 hours. The chloroform is removed and the extract dissolved in about 75 cc. of ether. This solution is extracted with two 15-cc. portions of 5-percent potassium hydroxide saturated with sodium chloride. These portions are extracted with ether, and the combined ether layer is washed once with 1+10 hydrochloric acid and once with a saturated sodium chloride solution. The alkali-soluble extract is discarded.
>
> The ether is removed, the resin dissolved in 40 cc. of carbon tetrachloride, and the rotenone solvate crystallized and separated in the usual way. The carbon tetrachloride is removed from the filtrate and the residue is dissolved in 10 to 15 cc. of methanol. While warm this solution is placed in a 25-cc. Erlenmeyer flask and 10 drops of 40-percent potassium hydroxide added. The contents are swirled and the flask is filled with warm methanol. A one-hole stopper carrying a funnel made from a drawn-out test tube is immediately inserted so that no air bubbles remain in the flask and some of the colorless liquid is forced up into the funnel. More methanol is poured in the funnel to allow for contraction on cooling and for evaporation. The solution is kept at about 45° C. for an hour to prevent separation of resin before it is racemized. If deguelin is present, crystals soon separate, but racemization is usually not complete until the material has stood overnight.
>
> The flask is then cooled at 0° C. for 1 hour. The methanol is then decanted through a small filter and the residue allowed to drain as completely as possible. For purification the deguelin crystals are dissolved in a little chloroform, and the chloroform is replaced by evaporating to a thick solution twice with carbon tetrachloride. Finally, the deguelin is crystallized from 5 or 10 cc. of carbon tetrachloride. It is usually

necessary to seed the solution with the carbon tetrachloride solvate of deguelin at 0° C. and let it stand overnight for complete crystallization. The crystals are then filtered on a tared Gooch crucible, washed with cold carbon tetrachloride saturated with deguelin, air-dried at room temperature for 4 hours, and weighed as the 1:1 deguelin-carbon tetrachloride solvate.

The amount of deguelin in this impure solvate is determined by the red color test (40). It is assumed that deguelin alone is responsible for the color, and the fact that racemic deguelin gives only 80 percent of the color given by rotenone is taken into consideration when rotenone is used as the standard of comparison. The effect of solubility in the solvents used is compensated for by adding 0.08 percent when 5 cc. of carbon tetrachloride is used for crystallization and 0.11 percent when 10 cc. is used.

The accuracy of the method was checked from several angles and it appeared that no great error was introduced. The possibility of decomposition of deguelin during the racemization was studied by using a concentrated sample of active deguelin prepared by high-vacuum distillation. Upon racemization of this material, 83 percent of inactive deguelin was obtained. Hence not more than 17 percent was believed to be destroyed in the racemization and probably much less, as the active deguelin was not pure. The precision was said to be equal to that of the rotenone analysis (72). Results for deguelin by this method were markedly lower than those by either the red color test (40) or the dehydro method (120). For example, on 13 samples of derris and cube values by racemization ranged from less than one-tenth to about four-tenths of those by the red color test. It was pointed out that some of this difference might be due to compounds other than deguelin that give the color test or dehydro derivatives.

As in previous work on the evaluation of rotenone-containing plants, Martin (83) in 1940 made quantitative determinations of the amounts of "deguelin concentrate" in several samples of derris. The method was similar to that employed in the earlier investigation (84) with certain modifications. The roots were extracted by percolation with ethyl acetate for 3 hours instead of by prolonged extraction with petroleum ether in a Soxhlet. Alkali extraction of the resins in ether solution was carried out before crystallization of the rotenone. In the case of roots rich in rotenone a mixture of ether and benzene was used as solvent for the extract during the alkali extraction. This scheme, which prevented crystallization of the rotenone during the extraction, has already been mentioned in connection with the determination of rotenone (59). After separation of the rotenone as the carbon tetrachloride solvate, the residual resin was designated the "deguelin concentrate." It was also stated that an assessment of the "deguelin concentrate" was obtained simply by subtracting from the percentage of "neutral resin" (alkali-insoluble material) the percentage of purified rotenone.

Discussion

The Durham color reaction (75) has become established as a rapid qualitative test for rotenone and related compounds. Likewise the red color test originated by Gross and Smith (50), particularly in the Goodhue modification (40), is widely used in the quantitative evaluation of material containing these substances. Since both reactions probably depend essentially on the same structural grouping, the substances present in derris and cube which will give one of these color reactions will probably give both. The dehydro reaction has not been used so widely as the color tests. Both the dehydro reaction and the color tests have been suggested as means of evaluating the toxicity of rotenone-containing plants.

At one time it was thought that rotenone and deguelin were the only substances present in derris and cube which were determined by these reactions, but there is now increasing evidence that this is not strictly true. Thus, although Haller and LaForge (55), Worsley (132), Tattersfield and Martin (120), and other investigators obtained comparable results on many samples by the dehydro method and by the red color test, on many other samples it has been found that the methods do not agree. For example, on a series of derris roots Jones (68) obtained results that were substantially lower by the dehydro method than by the red color method, while results for cube roots were comparable. It thus appears that in many samples substances are present which do not give both reactions, or at least not to the same degree. Furthermore, new compounds have been found in derris which both give the red color test and form dehydro derivatives (11, 56, 88). The amounts present of these other compounds have been stated to be small. The recent work of Goodhue and Haller (41), however, presents the possibility that large amounts of substances other than rotenone and deguelin may be determined by both the red color test and the dehydro reaction. On the other hand, it is possible that the deguelin may not all be present in such a form as to be determined by the racemization method. The earlier work of Haller and LaForge (55) had already indicated that not all the deguelin was present in a single form. Only further work can give a clear understanding into the cause of the considerable discrepancies between this and the earlier methods. In the meantime results by any of these methods should be considered as essentially empirical.

TOXICAROL AND OTHER ALKALI-SOLUBLE SUBSTANCES

In 1935 Jones, Campbell, and Sullivan (69) determined the amount of alkali-soluble material in several samples of derris and cube roots following the method used by Haller and LaForge (55) in preparative work. This involved extraction of an ether solution of the extract with 5-percent aqueous potassium hydroxide until no more material was dissolved. The alkaline layer was acidified and extracted with ether, and the extract was evaporated and weighed.

Martin and Tattersfield (84) in 1936 used essentially the same method for separating alkali-soluble and alkali-insoluble fractions of derris root. In one test the separation was made on an extract from which the rotenone had been crystallized; in the other the whole extract was used. In the latter case 5-gm. samples of root were extracted with ether and the ether solution, made to 50 cc., was extracted successively with 10, 5, and 5 cc. of 5-percent aqueous potassium hydroxide. In the case of Sumatra-type and *Derris malaccensis* extracts precipitates formed in the alkaline extract. The alkaline extract of *D. elliptica* resin showed no precipitate and very little alkali-soluble material. The resins from the precipitates and alkali-soluble fractions were recovered by acidification with dilute hydrochloric acid and solution in ether. The ether extracts were washed, dried with anhydrous sodium sulfate, evaporated, and dried to constant weight. Methoxyl determinations were made and the contents of "active principles," based upon the methoxyl content of toxicarol, were calculated. In one series of tests saturated barium hydroxide was substituted for potash. The amount of alkali-insoluble material agreed with that obtained by the use of potash, but the barium hydroxide extraction precipitated a larger proportion of the alkali-soluble material than did potash. The fractionation with aqueous potash was repeated using benzene instead of ether as solvent for the extract, but only a relatively small percentage of the resin was extracted by the alkali from the benzene extracts of the three types of derris root examined. Potash did not effect complete separation of the potassium salt of toxicarol from a benzene solution of Sumatra-type or *Derris malaccensis* resins.

Cahn, Phipers, and Boam (17) in 1938 described a color test for the determination of toxicarol based on the deep green color obtained with ferric chloride.

> "One drop (0.06 cc.) of a 5 percent aqueous solution of commercial ferric chloride (hydrated) is added to 10 cc. of alcohol containing an amount of Derris extract approximately equivalent to 1 mg. of toxicarol; the color developed is matched in a Duboscq colorimeter against that from a standard solution of exactly 1 mg. of toxicarol in 10 cc. of alcohol. The approximate amount of extract to be taken may be determined by rough visual matching, using solutions containing 5 mg. of toxicarol or its equivalent in 10 cc. of alcohol. The color is permanent for quite long periods."

It was found that absolute or 96-percent alcohol could be used. Pure ferric chloride offered no advantage over the commercial grade. Ferrous sulfate gave no color, while ferric alum gave only a slight color. The size of the drop was determined by the dropping pipette arbitrarily chosen; a similar drop of 2.5-percent ferric chloride solution gave a slightly weaker color, but 1 and 2 drops of the 5-percent solution gave identical colors. Within limits, therefore, the amount of ferric chloride appeared to be immaterial provided sufficient was used. The alcoholic solution could contain at least 3 percent of acetone, benzene, or chloroform without interfering with the results. Extracts of Sumatra-type roots gave a deep green ferric chloride color and were easily matched against pure toxicarol. However, as the rotenone content of an extract increased the ferric chloride color became more and more brown, and with rotenone-rich extracts matching in the Duboscq colorimeter was very uncertain. This difficulty was overcome by employing secondary standards. Thus it was found that extracts containing less than 20 percent of rotenone could be matched against Sumatra-type extracts. One particular Sumatra-type extract was accordingly taken as a subsidiary standard. For extracts containing more than 20 percent of rotenone even this was not satisfactory. Ethereal solutions of such extracts were shaken with 5-percent aqueous potassium hydroxide and the phenols recovered from the alkaline layer and separated solid salts (if any) by acidification and extraction with ether. The ferric chloride value was then determined on this alkali-soluble part only.

The authors proposed the term "ferric chloride value" to denote the percentage of toxicarol that the sample would contain if the intensity of color developed was due entirely to toxicarol. Although sumatrol gave a brown color with ferric chloride, this color was believed to contain a green component which contributed to the total ferric chloride color. Consequently, the ferric chloride values of derris extracts were interpreted as giving the sum of the percentage of toxicarol and sumatrol. However, the yield of sumatrol isolated from derris was said to be always much less than the yield of toxicarol. Other substances giving a green color might also be present in derris, but they had not been isolated up to this time.

Rowaan and Van Duuren (106) in 1938 described a quantitative method for the determination of toxicarol based on the earlier qualitative separation of this substance by Clark (21). It was as follows:

> The carbon tetrachloride filtrate from the rotenone determination is freed of solvent and the residue dissolved, by warming, in 100 cc. of 95-percent ethyl alcohol. After the addition of 10 cc. of 1N. sodium hydroxide the solution is boiled. On cooling, the separated sodium salt of toxicarol is filtered, washed with alcohol, dried at 100°- 105° C., and weighed. The weight multiplied by 0.95 gives the weight of toxicarol.

The ferric chloride color test of Cahn and his coworkers (17) was used by Jones (68) in 1939 in the examination of a series of derris and cube roots. Determinations were made by the method already described including

the use of secondary standards where necessary. Difficulty was encountered in matching the colors from the cube samples, and the use of the alkali-soluble fractions of the extracts did not improve the color matching. The amount of alkali-soluble material was determined by the method previously used (69).

Martin (83) in 1940 made determinations of alkali-soluble material, or of "toxicarol fraction," on several samples of derris. The method was similar to that used in previous work (84) except that extraction of the ether solution was made first with one lot of 2-percent and then with two lots of 5-percent potassium hydroxide. As mentioned under the sections on Rotenone and Deguelin, a mixture of ether with 25 percent of benzene was employed as the solvent for extracts of high-rotenone roots. It was also stated that the percentage of "toxicarol fraction" removed by the potash could be determined from the difference in the percentages of original and "neutral" (alkali-insoluble) resins.

Discussion

Both the determination of alkali-soluble material and the ferric chloride color test must be considered to give only empirical values. It is probable that in Sumatra-type derris roots both these methods give reasonably close approximations of the toxicarol plus sumatrol content. In other samples of derris and particularly in cube samples large amounts of interfering substances are undoubtedly present. Since the ferric chloride test was proposed an additional phenol, malaccol, has been isolated from derris (88). Rowaan and Van Duuren (108) state that toxicarol has not yet been isolated from Lonchocarpus (cube), and attempts to isolate this compound from cube root in the laboratories of this Bureau have been unsuccessful. Nevertheless, the samples of cube examined by Jones (68) gave ferric chloride values equivalent to 1.5 to 2.8 percent of toxicarol, presumably due to other phenolic bodies, and contained 2.8 to 5.0 percent of alkali-soluble material.

The method of Rowaan and Van Duuren (108), based on the separation of inactive toxicarol, should be subject to less error from interfering substances. However, the accuracy of the scheme may be questioned when only small amounts of toxicarol are present.

MISCELLANEOUS COLORIMETRIC DETERMINATIONS

Sulfuric Acid-Nitrite Color Reaction

In 1899 Sillevoldt (114) found that his "derrid," which undoubtedly contained a high percentage of rotenone, gave a brown-violet color with concentrated sulfuric acid. Danckwortt, Budde, and Baumgarten (25) in 1934 found that sulfuric acid followed by a small amount of sodium nitrite gave an intense red-violet color with rotenone. This finding suggests that Sillevoldt's acid contained a trace of nitrite. When the test was applied to derris root, a 0.5-gm. sample was shaken with 5 cc. of chloroform and 1 to 2 drops of the filtered extract were evaporated on a watch glass. To

this residue was added 6 to 8 drops of concentrated sulfuric acid and a crystal of sodium nitrite. The red-violet color developed in from 1/2 to 5 minutes. A similar result was obtained when the test was carried out in the same way but with water instead of chloroform. The color was also given by dehydrorotenone, dehydrodeguelin, and isodihydrodehydrorotenone. Toxicarol was said to give a red-brown color only.

This color reaction was developed into a quantitative test by Fischer and Nitsche (28) in 1935. The method was essentially as follows:

> One cubic centimeter of an acetone solution containing 0.2 to 0,5 mg. of derris resin is placed in a 25-cc. volumetric flask and evaporated to dryness. The residue is cooled and dissolved in 2 cc. of concentrated sulfuric acid, and 5 drops of a 1-percent solution of sodium nitrite in concentrated sulfuric acid are added. The flask is heated on the water bath for 8 minutes, cooled, and the solution is made to volume with concentrated sulfuric acid. Comparison is made in a colorimeter with the color developed from a standard solution of rotenone.

Pure rotenone gave a "permanganate-like" violet, while derris resins gave a more reddish violet. Since the authors used an "absolute" colorimeter, it was not necessary to repeat the rotenone standard. A red filter was found to be suitable in the color measurement, although a green filter was sometimes used. Acetone, benzene, and chloroform extracts gave equal color values. Ether extracts, however, gave erratic results. Too much nitrite was found to destroy the color. Toxicarol gave a color reaction equally as strong as did rotenone but of a more reddish violet. The mixture of dehydro compounds obtained in the Takei (118) method gave a color exactly like that of rotenone in tone and intensity.

The sulfuric acid-nitrite test was modified by Meijer (85) in 1936. In this form an aqueous suspension of the material to be tested was treated with the reagent. The method was designed primarily for the rapid determination of total ether extract and was said to require about 20 minutes. It was as follows:

> A 1-gm. sample of derris root is extracted by shaking in a test tube with 10 cc. of acetone for 5 minutes. One cubic centimeter of the filtered extract is diluted to 25 cc. with distilled water. This milky suspension is well shaken, and 0.2 cc. in a dry test tube is treated with 5 cc. of a solution of sodium nitrite in concentrated sulfuric acid containing 10 mg. of sodium nitrite per 100 cc.

The addition of the sulfuric acid to the aqueous suspension caused sufficient heat to produce the maximum color. The violet color obtained was measured in a step photometer with a 5300 A. filter. The results were compared with determinations made on samples of known total-extract content. The relation between extinction coefficient at 5300 A. and percentage of ether

extract followed Beer's law. It was found that the color could also be compared visually with permanent color standards prepared from cobalt chloride. The standards were made by mixing in various proportions a 10-percent solution of the cobalt salt in 96-percent alcohol, a 10-percent solution of the cobalt salt in water, and 96-percent alcohol. When kept in sealed tubes these color standards were stable for a long period. The color reaction required a strongly acid medium. Seventy percent trichloroacetic acid instead of sulfuric also gave the color but with a more reddish shade. Acetic, oxalic, and tartaric acids gave no color. Hydrochloric acid gave only a faint pink color. Since the variety of derris known in the Dutch East Indies as "Toeba woeloeng" had previously been shown to have a definite ratio of rotenone to total extract, it was suggested that this method would give approximate values for rotenone in this type of root.

Goudswaard and Timmers (43) in 1937 stated that this reaction could be used in the inverse sense for the detection of nitrates and nitrites in sulfuric acid. Sulfuric acid that conformed to the Dutch pharmacopoeia contained sufficient nitrites to give a color with rotenone. In the usual form of the test the color developed was proportional to the rotenone present and was stable for 24 hours. The test was said to be unsuitable for the estimation of rotenone in derris root because the reaction was not specific for rotenone.

Cahn, Phipers, and Boam (17) in 1938 found that the Meijer color test was given by many substances other than derris extract; consequently, the method was applicable only in the absence of interfering substances and when the genuineness of the root was certain. The color was given with equal intensity by rotenone, deguelin, toxicarol, and sumatrol, as well as by many of their derivatives. All derris extracts tested were found to give the color with about 90 percent of the intensity given by rotenone. A modification of the Meijer test was used in this work (private communication).

In 1939 Jones (68) used the sulfuric acid-nitrite test in the form described by Meijer, and made color comparisons with a rotenone standard in a Duboscq type of colorimeter without a filter. Values for the derris samples averaged about 90 percent of the extract, but those for the cube samples were lower. The test was said to give a rough estimate of the total materials of the rotenone type.

The Rogers and Calamari Test

Rogers and Calamari (102) found in 1936 that in the presence of hydrochloric acid and certain phenols rotenone developed colors ranging from blue to violet-red depending upon the solvent and the phenol used. Such organic solvents as chloroform, ethylene dichloride, carbon tetrachloride, ether, alcohol, and acetone were used. Phenol, guaiacol, and thymol reacted similarly in these solvents. Small amounts of hydrogen peroxide and nitric acid and light exerted a marked influence in accelerating the reaction. The color was also given by certain derivatives of rotenone. Both qualitative and quantitative tests based on this color reaction were developed. Substances usually found in proprietary liquid insecticides, such as pyrethrum extract, aliphatic thiocyanates, and oil of sassafras, were said not to interfere with the tests. In the qualitative test a chloroform solution of the sample was treated with a chloroform solution

of thymol. A mixture of 0.2 part of concentrated nitric acid and 100 parts of hydrochloric acid was then added and the solution shaken. A blue-green to blue color appeared in from 30 seconds to 2 minutes when rotenone was present. The following quantitative test was designed for use with colorless liquid insecticides:

> To 10 ml. of a chloroform solution containing from about 0.05 to 2.5 mg. of rotenone per milliliter in a glass-stoppered cylinder, add 10 ml. of a chloroform solution of thymol (10 gm. of thymol to 100 ml. of chloroform) and 2 ml. of a reagent made by adding 2.5 ml. of 3-percent hydrogen peroxide to 100 ml. of concentrated hydrochloric acid. (When the sample is not in solution in a hydrocarbon base, concentrated hydrochloric acid may be used in place of this reagent.) Agitate for 1 minute, loosen the glass stopper, and expose the cylinder to the intense radiation of a quartz-mercury vapor lamp (minimum output 1000 microwatts per square cubic centimeter in the field of exposure). A greenish-blue color appears in the chloroform layer in about 15 minutes. (Exposure to bright sunlight produces similar results in about 3 hours.) At the end of 30 minutes compare with standards containing known quantities of pure rotenone prepared simultaneously in the same way. If the insecticide base is refined kerosene, use an equal amount of refined kerosene in preparing the standards.

A more rapid method making use of an acetone solution was as follows:

> To 5 ml. of an acetone solution containing 0.1 to 2.0 mg. of rotenone per milliliter add 5 ml. of an acetone solution of thymol (10 gm. of thymol to 100 ml. of acetone), 0.1 ml. of 3-percent hydrogen peroxide, and 5 ml. of concentrated hydrochloric acid. A reddish-violet color appears within 30 seconds. After 1 minute place the cylinder in a water bath at about 20° C., and at the end of 20 minutes compare with rotenone standards similarly prepared at the same time.

If the liquid extract contained pyrethrum extract, it was recommended that a standard containing pyrethrum extract be prepared to match the original color of the liquid insecticide.

Cahn and Boam (14) in the same year reported failure to obtain the Rogers and Calamari qualitative test with the rapidity and intensity stated by the authors of the test. These factors were found to be markedly affected by the nitrous acid content of the nitric acid. With a mixture of fresh nitric and hydrochloric acids the rotenone color was produced only slowly. When nitrite was added to the mixture, or when the acid mixture was allowed to stand before use, the rotenone color developed rapidly. It was concluded that the reaction depended more upon the presence or production of nitrous acid than upon the presence of nitric acid.

In their reply Rogers and Calamari (103) stated that it was essential to use concentrated hydrochloric acid containing not less than 35 percent

of hydrogen chloride and pointed out that Cahn and Boam used an acid containing only about 30 percent of hydrogen chloride. They found that when the stronger hydrochloric acid was used the nitrogen peroxide content of the nitric acid was unimportant. The intensity and speed of the color reaction depended on the concentration of the acid used. They believed that the acid reagent depended on the formation of nitrosyl chloride for its action and that the reagent should stand at least 3 minutes before use.

Cahn and Boam (15) in 1937 reported that when they used 36.45 percent hydrochloric acid (sp. gr. 1.18) their results were in substantial agreement with those of Rogers and Calamari. They found, however, that even then the rate of development of color and its final intensity increased greatly as the acid mixture was allowed to stand before use. They emphasized that the sensitivity of the reaction depended greatly on the exact conditions used.

According to DeOng (27) the Rogers and Calamari method proved unsatisfactory for the quantitative determination of rotenone in fly sprays.

In 1939 Jones (68) modified the Rogers and Calamari test for the analysis of several samples of derris and cube roots. The color produced in the acetone test was not proportional to the rotenone present. In chloroform solution the proportionality between color and concentration held. Heat as well as light accelerated the color formation, but the latter was adopted. The solutions were exposed in glass cylinders to daylight (not direct sunlight) for 24 hours. Perchloric acid was found to effect a more rapid development of the color than hydrogen peroxide. At a rotenone concentration of 0.12 mg. per cubic centimeter, 2 drops of 60-percent perchloric acid gave a moderately intense, pure-blue color in 24 hours. Even in this form results were erratic and duplicate standards varied as much as 10 percent. Deguelin was found to give a color intensity about 125 percent of that given by rotenone, whereas toxicarol gave only about one-half the rotenone color. When rotenone was used as a standard values for the derris roots ranged from 60 to 120 percent, and those for cube and timbo from 140 to 160 percent of the total extractives.

The method of Rogers and Calamari was recently used as a qualitative test for rotenone in miscellaneous insecticides (127). The characteristic blue color did not develop when too much rotenone was present; therefore, in case of failure to obtain the test a repeat should be made with a smaller sample.

Other Color Reactions

Geoffroy (29) in 1895 was the first to study the color reactions of rotenone with several reagents. The most characteristic involved treatment with bromine, followed by application of concentrated sulfuric acid to the residue. A violet color was produced.

Dennis (26), in a patent on cube issued in 1927, described a color reaction for testing the material using sulfuric and nitric acids followed by potassium hydroxide. Jones (68) in 1939 found that this test gave identical color reactions with roots of derris, cube, and *Tephrosia virginiana*.

Schmitt (109) in 1930 stated that rotenone may be detected in ground root by treatment with nitric acid, whereupon the affected portions turned red.

Pozzi-Escot (95) in 1936 stated that rotenone dissolved in concentrated sulfuric acid with the formation of a red-cerise or rose-cerise color. When the acid contained mercuric oxide in solution, as in Denigé's reagent, the color reaction with rotenone was from 100 to 1,000 times more sensitive and was not subject to interference from as many other substances as when acid alone was used. On being warmed with this reagent, the rotenone dissolved giving an intense orange-yellow color. The intensity of color was observed to be proportional to the amount of rotenone.

In later work Pozzi-Escot (98) discussed the color reactions given by sulfuric acid, sulfuric and nitric acids, and Denigé's reagent with substances that might interfere with the rotenone reaction, and described methods for differentiating between the reaction of rotenone and reactions of other substances.

In 1937 Tapia Freses (119) reported that rotenone gave a red-violet color with a solution containing 0.1 gm. of vanadium pentoxide in 10 cc. of sulfuric acid.

OTHER DETERMINATIONS

Polarimetric Methods

Danckwortt and Budde (24) in 1933 stated that polarimetric determinations might ultimately be used for the evaluation of derris root.

In the same year Jones (62) briefly studied the possibility of using the high optical rotation of rotenone (74) (in benzene $[\alpha]_D^{20°} = 224°$ for a 5-percent solution) as a means for its determination. The optical rotatory powers of derris and cube extracts were determined in several solvents, and a hypothetical rotenone content was calculated from the values for pure rotenone. In most cases the results were very much higher than those obtained by crystallization. Since one derris extract was dextrorotatory, it was concluded that optical rotation cannot be used as a measure of the amount of rotenone present in the root.

A polarimetric method proposed by Danckwortt, Budde, and Baumgarten (25) in 1934 was claimed to be superior to the crystallization methods, at least as a measure of the toxicity of derris. Directions were as follows:

> 3 gm. of finely pulverized sample is digested with 30 cc. of benzene at room temperature for 24 hours. The optical rotation of the filtered benzene solution is determined in a 100-mm. tube. The rotenone content is obtained by the following formula:

$$\text{Percent rotenone} = \frac{\alpha \times 1{,}000}{233}$$

In 10 out of 11 samples of derris root the method gave values higher than those by crystallization. The method was also adapted to the deter-

mination of rotenone in aqueous extracts of derris, and this use of the procedure was described in detail.

Ostirner (51) in 1934 employed the method of Danckwortt and coworkers in the analysis of 19 samples and fractions of samples of derris root. In all cases except one results were higher than those by crystallization, in most cases markedly higher. This difference was attributed to the rotenone remaining in the resin from the crystallization.

Danckwortt's method was also used by Fischer and Nitsche (28) in 1935 in a study of chemical methods for the evaluation of commercial derris preparations. These investigators found that extraction of the sample for this purpose was complete in 3 to 4 hours. In certain cases it was necessary to have more derris resin present than specified by Danckwortt and coworkers.

Rowaan (105) in 1935 reported that in analyses of samples of derris root the rotenone content was usually higher by the polarimetric method of Danckwortt and his coworkers than by crystallization, but that in two samples the values by crystallization were about double those by the optical-rotation method. Rowaan also reported the isolation of a dextrorotatory preparation of toxicarol from derris. Because of these circumstances analysts were warned against the use of the polarimetric method.

In reply Danckwortt (23) pointed out that the polarimetric method was a means of determining the effective value of derris and not of determining rotenone. He also reported the use of the method in a study of the relative stability of industrial derris extracts over a period of time. The extracts were dried with sand, the residue was shaken with benzene for 20 minutes, and the optical rotation of the solution was determined as previously described. Strongly colored solutions were shaken with animal charcoal. Too much charcoal was avoided, since it caused a decrease in optical activity.

Rowaan (106) in 1936 again warned against the use of the polarimetric method for the determination of rotenone in derris and stated that the most reliable method was some modification of the extraction-crystallization method.

Tattersfield and Martin (121) in 1936 studied the optical activity of benzene solutions of resins and their fractions derived from samples of Derris elliptica, D. malaccensis, and Sumatra-type derris root. A fraction of Sumatra-type resin obtained by means of methyl alcohol was dextrorotatory in methyl alcohol but levorotatory in benzene.

A study of the optical rotatory power of acetone and benzene extracts of 16 samples of derris and 10 samples of cube root was reported by Jones (65) in 1936. Calculations of hypothetical rotenone contents were based on the known rotations of pure rotenone in acetone and benzene, as was done in earlier work (62). All extracts were levorotatory in benzene, but

acetone extracts of certain derris roots with little or no rotenone by crystallization were dextrorotatory. There was no agreement between the value obtained by crystallization and the values obtained from the optical rotatory powers of either acetone or benzene extracts. A further calculation based on optical rotatory power which used the rotations of both acetone and benzene extracts was made by Jones. It was based on the assumption that the optical rotatory power of all the rotenonelike compounds present was greater in benzene than in acetone to the same degree as was that of rotenone, and that the rotation of the oth r optically active material was the same in both solvents. All values by this method were markedly higher than values obtained by crystallization. It was concluded that the optical rotatory power could not be recommended as a means of evaluation of derris and cube root.

One of the laboratories participating in the collaborative analysis of derris reported by Koolhaas and Meijer (see p. 26) in 1937 used the polarimetric method of Danckwortt. The use of this method for the determination of rotenone was criticized by the authors of the report because of the general lack of knowledge of the significance of optical activity and of the proportion of other constituents present.

Worsley (132) in 1937 studied the use of optical rotatory power as a means of assessing the toxicity of derris root. The dried extract from hot percolation of the root with ethyl acetate was dissolved in benzene and the concentration of the solution adjust d so that the angle of rotation in a 200-mm. tube was about 22 degrees. Concentrations between 25 and 200 gm of original sample in 25 cc. of benzene were involved. Direct extraction with benzene in a Soxhlet gave the same results. From a curve for the optical rotation of pure rotenone a value was read off corresponding to the angle obtained as described in Worsley's method for rotenone determination (130). Because in most samples this value agreed rather closely with that for dehydro compounds, Worsley used the term "optical dehydro compounds." In a subsequent article Worsley (133) proposed the term "optical constituents " instead of "optical dehydro compounds" for the value described in the previous work. He also proposed the use of the reciprocal of this value for toxicity comparisons.

Guillaume and Proeschel (54) in 1937 stated that the polarimetric method was used in Germany, but that investigators in other countries found it gave too high results.

Rowaan and Van Duuren (108) in 1938 confirmed in general the observations of Jones (65) on the optical activity of derris and cube extracts, particularly with regard to the dextrorotation of derris extracts that gave no rotenone by crystallization.

Thung (123) in 1939 stated that in the analysis of derris powder a 2-gm. sample was extracted by shaking repeatedly with 53 cc. of benzene during 24 hours. The optical rotation of the filtered extract was determined in a 200-mm. tube (0.5° V. = 1 percent rotenone).

Determination of Methoxyl Content

Tattersfield and Roach (122) in 1923 stated that the genuineness of derris extracts might be confirmed by the determination of their methoxyl content. Determinations were made on the dried ether extractives by Perkin's modification (91) of the Zeisel method. The methoxyl content of different samples of extract ranged between 13.5 and 14.7 percent.

Danckwortt and Budde (24) in 1933 determined the methoxyl content of benzene extracts of derris root.

In 1934 Danckwortt, Budde, and Baumgarten (25) described their method for the determination of the methoxyl content of derris extract. The benzene extract was transferred directly to the flask of a Zeisel methoxyl determination apparatus and evaporated to dryness therein. The usual Zeisel method was used. One gram of silver iodide corresponded to 0.6394 gram of "active substance," calculated as rotenone. This determination was said to have only an orienting value.

Campbell, Sullivan, and Jones (18) in 1934 determined the methoxyl contents of derris and cube roots by the method of Vieböck and Schwappach as modified by Clark (22). The determinations were made on acetone extractives, which had been treated with benzene to remove traces of acetone. In 1935 these investigators (69) used the same method on benzene extracts of derris and cube and calculated the results to material having the same methoxyl content as rotenone. These values were said to be significant, as all the known naturally occurring compounds of the rotenone group had approximately the same methoxyl content as rotenone.

Methoxyl determinations were made by Tattersfield and Martin (120) in 1935 on samples of three species of Derris. Values were determined on dried ether extracts by the method of Clark (22). The results were closely correlated with the weights of the ether extract.

Martin and Tattersfield (84) in 1936 found the methoxyl content of the ether extracts of three samples of derris root in good agreement with that of the benzene extracts. They also made methoxyl determinations on various fractions of the extracts.

Guillaume and Hervé (53) in 1939 used a modification of the Zeisel method in determining the methoxyl content of derris, cube, and other rotenone-containing materials. The methyl iodide formed was run into 0.1 N silver nitrate and the excess of silver nitrate was titrated with 0.1 N sodium thiosulfate. The value obtained was calculated to material of the same methoxyl content as rotenone. The results, in the 11 samples tested, were in good agreement with those by the modified Durham blue color test used by these investigators.

Miscellaneous Determinations

Danckwortt and Budde (24) in 1933 stated that attempts to measure the absorption of iodine or bromine by the double bond of rotenone as a method of determination led to practical difficulties. Again in 1934 they reported (25) that, since the halogen consumption was dependent on factors other than

the quantity of rotenone, a reproducible method could not be attained.

Whittaker and Glickman (129) in 1934 described a method for the determination of rotenone which was an adaptation of the method of Gnadinger and Corl (39) for the pyrethrins. It involved reduction of an alkaline copper solution, a modified Folin's solution, by standard solutions of dextrose and of rotenone and by the unknown solution of rotenone. The reagents and procedure were almost identical with those described by Gnadinger and Corl. A standard rotenone solution in 95-percent ethyl alcohol was used. Results for standard rotenone solutions ranged from 9.60 mg. of rotenone equivalent in reducing action to 1.25 mg. of dextrose to 21.09 mg. of rotenone equivalent to 4.19 mg. of dextrose. From these results an equation was obtained for calculating the amount of rotenone present from the equivalent amount of dextrose. In a second series of tests the results agreed well with those calculated from this equation. The precision of the method was said to be usually about 2 parts per thousand. Traces of chloroform and carbon tetrachloride interfered with the determination.

This procedure was successfully used for the determination of rotenone in alcoholic solutions containing antioxidants. When the method was applied to the analysis of derris root, the results were much higher than those obtained by the crystallization method. For example, two samples which gave about 6 percent of rotenone by crystallization gave 11.5 and 15.5 percent by the reduction method. A sample with no rotenone gave 15.5 percent by the present method.

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